Blood Cell Morphology: Controversies and Alternatives

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Chapter 1

General introduction

1. Preface

This thesis deals with the controversies in both microscopic and automated blood cell morphology. Since Anthony van Leeuwenhoek discovered blood cells with his newly discovered microscope, blood cell differentiation is a world wide accepted diagnosticum, and has developed to full-automated recognition of blood cells nowadays *(figure 1).*

In this thesis we describe some controversial morphologic features of various cells and we offer alternatives. Particularly, with respect to sepsis diagnosis. In some cases, a microscopic observation of blood smears is necessary and may lead to a better understanding of haematology analysers and their automated differentials.

fig 1

a Van Leeuwenhoek's microscope

b Fully automated microscope DM96 of Sysmex

2. Differentiation of blood cells

In one litre human blood 3 to 5 trillion red blood cells, 4.5 to 12 billion white blood cells and 120 to 350 billion platelets circulate. To diagnose various diseases, e.g. anaemia, infection, or bleeding disorders, these cells are counted using automated analysers. In addition, a blood smear can be made to analyse morphologic features of the cells in case of diseases as haemoglobin-, neoplastic- or platelet disorders. Differentiation of white blood cells plays an important role in diagnosis and follow-up of various haematological and non-haematological diseases. Therefore a large number of differentials are requested daily in laboratory medicine[1]. In the blood, mature cells are circulating which are released from the bone marrow. Five major white cell populations can be distinguished: monocytes, lymphocytes, segmented neutrophils, eosinophils, and basophils. The composition and/or the number of these cells can be indicative for various diseases. In order to analyse these cells microscopically, blood smears have to be made and stained to

distinguish the different cells. Worldwide two staining methods are used: Wright staining and May-Grünwald Giemsa staining. Both methods enable to differentiate white blood cells based on size, nuclear shape and density, cytoplasmic colouring and granularity. For routine microscopic differentiation of white blood cells one hundred cells are differentiated and reported as percentage and/or absolute counts. Nowadays, instead of micrsocopic differentiation automated differentials are used. Automated differentials are based on flow cytometry, impedance technique or cytochemistry. Abnormalities may be recognised by automated analysers and flagged for. Microscopic control is than often necessary.

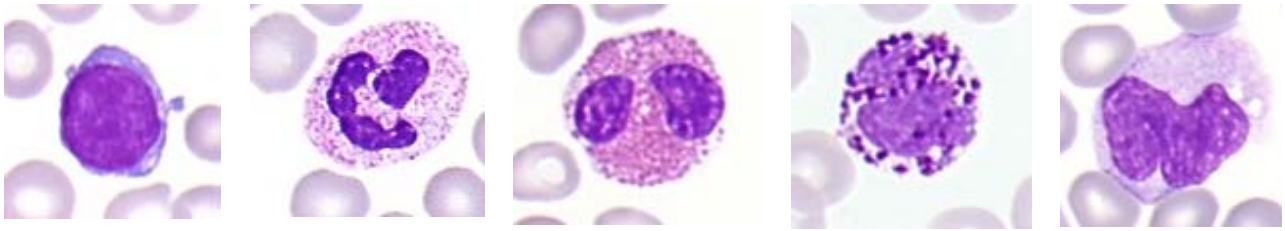


fig 2
a lymphocyte b neutrophil c eosinophil d basophil e monocyte

2.1 Microscopy

To identify different subtypes of leukocytes routine stains are used *(figure 2).* The most frequently used staining methods are the May-Grünwald Giemsa staining and the Wright staining method. With these staining methods blood cells can be differentiated into neutrophils, lymphocytes, monocytes, eosinophils, basophils and their precursor cells. Moreover, platelets can be recognized and erythrocytes can be observed and checked for abnormalities.

For specific cell details and subclassifications other staining methods were developed e.g. peroxidase-, PAS- and sudan black staining. In the present time, flow cytometry is used to substitute these methods because of their sensitivity and specificity[2].

Enumeration and proportion of cell types can attribute to the diagnosis of several diseases. Nevertheless, differentiation of 100 or 200 cells have large sample variance. Moreover, quality of the blood smears and expertise of the observer play a major role in the reliance of the final result[3]. Despite these limitations, this method is still used as the golden standard[4, 5], while some abnormalities are not recognized by automated haematology analysers, even in some cases no flagging appear at all, e.g. the presence of a small amount of malaria parasites or bacteria. In Chapter two the presence of bacteria in blood smears is described. Normally the presence of bacteria in blood is demonstrated by

conventional blood cultures. However these results are often only available after several days. Sometimes bacteria are seen in routinely obtained blood smears and become sufficiently numerous to be identified in the terminal phase of an overwhelming sepsis[6-8]. The finding of intracellular bacteria in routine blood smears prepared from a blood sample taken from a central venous catheter (CVC) most likely indicates catheter related infections[6, 9-11]. Blood samples obtained by finger or heel prick may contain bacteria or fungi as a consequence of *in vitro* contamination. In these situations bacteria will only be present extracellularly. However, if a long period has elapsed from the time of the puncture to the moment of preparing the blood smear, phagocytosis by neutrophils may lead to intracellular bacteria. The finding of bacteria in blood smears may result in the identification of a severe sepsis, CVC related infection or an *in vitro* contamination. These findings indicate that microscopy may contribute to a fast diagnosis of overwhelming sepsis not yet determined by blood cultures[6, 12, 13].

2.2 Automated differentials

Over the past 30 years, continuing technology in cell counting and differentiation has challenged the microscopic differentials[14]. The current automated analysers are capable of providing multiparameter, white blood cell, red blood cell and platelet parameters. In the beginning of the seventies automated image processing were developed by several manufacturers (e.g. Corning, Abbott and Sysmex), and were based on imaging recognition software. The Hemalog D (Technicon), cell counting and differentials were combined. Elaborating the Hemalog D analyser several other manufacturers developed machines[15]. The next generation automated haematology analysers generated full blood cell counts, including a five-part differential. The used techniques varied form impedance, flow cytometry to peroxidase staining, or a combination of these methods[16-20]. Refinement of the technique resulted in compact automated instruments, which generated a full blood cell count including differentials and reticulocytes with additional parameters such as nucleated red blood cells[21-25]. Instruments can be linked to a full automated ‘street‘, including an automated slide maker and stainer. Methods are developed to add cell indices such as immature granulocytes, reticulated platelets, reticulocyte haemoglobin [26-29]. Even monoclonal antibodies can be used on a routine haematology analyser[30, 31].

The first automated analysers were able to detect abnormal cell populations and flagged for these abnormalities. Using these flaggings the number of microscopic differentials could be dramatically reduced.

However, these flaggings seemed to have poor sensitivity and specificity[32]. Chapter 3 describes the overestimation of abnormalities based on these flaggings. And, even worser, some abnormalities are not accompanied by flagging messages at all (e.g. bacteraemia, malaria; *figure 3*).

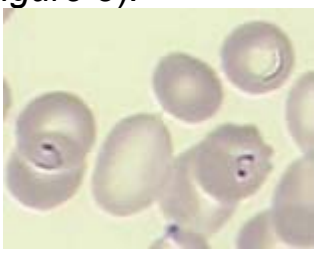


fig 3
Intracellular malaria parasites

However, traditional microscopy will continue to be used in routine haematology in combination with automated image recognition systems. Further advances in the technology of automated image recognition systems can be expected to create new challenges for the blood cell image interpretation[15].

2.3 Band cells

Upon activation of neutrophils, band cells *(figure 4)* appear in the circulation. Therefore enumeration of band cells is ingrained in clinical practice[33]. Although the descriptive definition of a band cell is well documented[34-36], the ability of individual laboratory technicians to interpret and apply these descriptive guidelines consistently is actually quite poor[37]. In addition to band cells, immature granulocytes defined as metamyelocytes and myelocytes, may be seen. Modern haematology analysers are able to generate automated five-part differentials (expressed in relative as well as absolute counts) and resolve many of the statistical limitations of the microscopic differential[14, 16]. Technical improvements have lead to expansion of the morphological subtypes of cell classes e.g. immature granulocytes[26] that can be recognized. Moreover, the general reliability of automated leukocyte differentials has significantly reduced conventional microscopic reviews. Flagging for morphological abnormalities, however, still has little sensitivity[32]. Therefore, flagging for abnormalities may lead to overestimation of the number of abnormalities.

Due to this statistical unreliability and the poor diagnostic value of the enumeration of band cells, new diagnostic tools have been developed to detect sepsis, such as expression of proteins and interleukins[38].

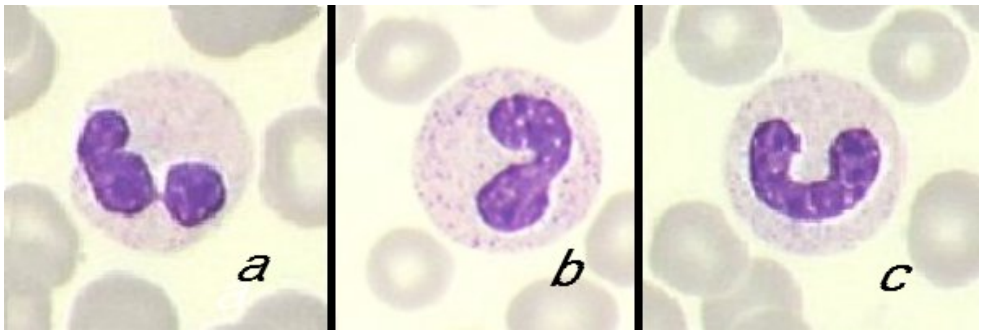
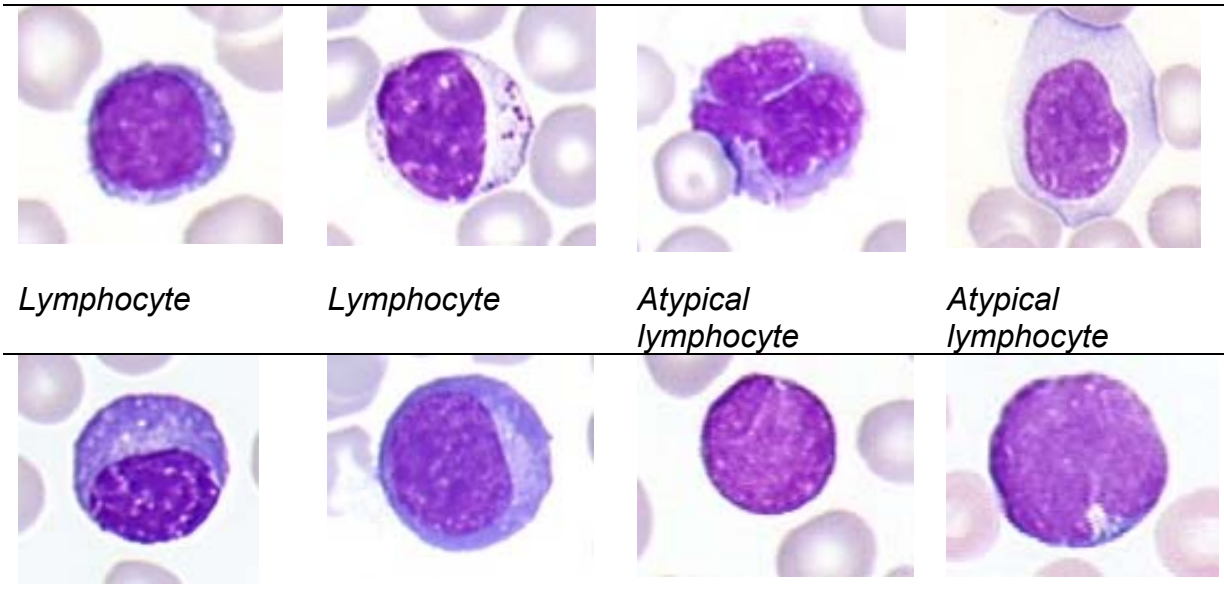


fig 4
a) Segmented neutrophil, b) controversial band cell, c) band cell

2.4 Atypical lymphocytes

The classification of lymphocyte disorders is complex because of the various manifestations of these disorders[39, 40]. Roughly three major populations can be distinguished: reactive lymphocytosis, premalignant neoplastic disorders and neoplastic disorders of lymphocytes[5]. Lymphocyte disorders can be accompanied by abnormal lymphocyte morphology. It is therefore of utmost importance to recognize abnormal lymphocytes, such as atypical lymphocytes and lymphoblasts. However, the ability of individual laboratory technicians to interpret abnormal lymphocytes consistently is quite poor. There are no standardised definitions and interpretation is based on subjectivity and depends on additional clinical information. Abnormal lymphocytes also can contribute to a quick diagnosis of various diseases, like viral infections and leukaemia, but must be interpreted with care. Some rare diseases are



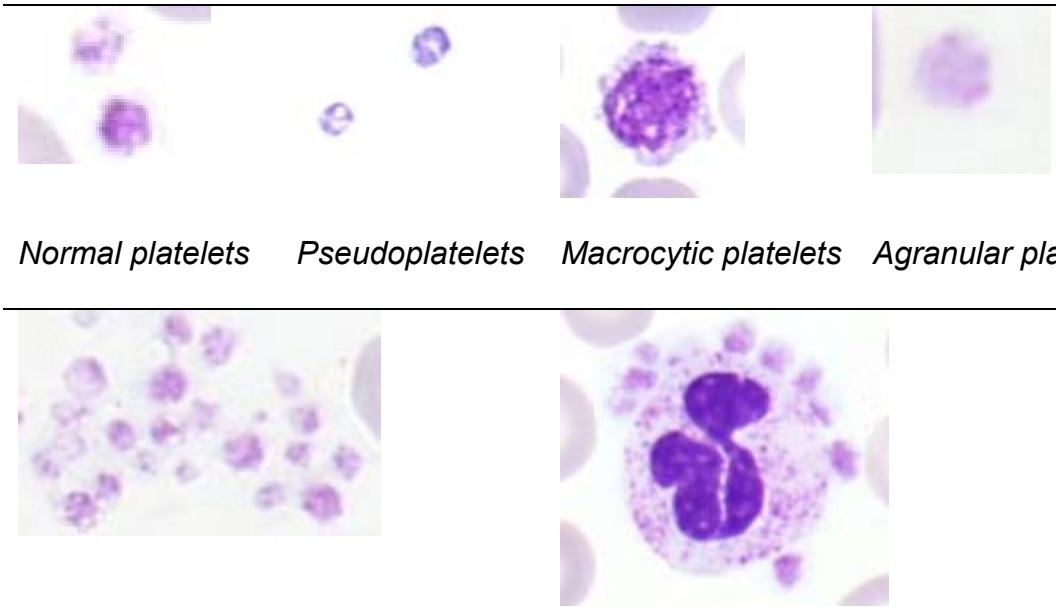
Lymphocyte	*Lymphocyte*	*Atypical lymphocyte*	*Atypical lymphocyte*
Plasma cell	*Plasma cell*	*Blast*	*Blast*

fig 5
Morphology of lymphocytes

accompanied with atypical lymphocytes. For I-cell disease, inclusion bodies are formed by accumulated macromolecules due to multiple lysosomal enzyme deficiencies, these are seen as vacuole like inclusions[41-43].
The problem to classify subtypes of lymphocytes gives us the opportunity to make two steps; microscopic classification of lymphocytes should be standardised and variant (or suspicious) lymphocytes (*figure 5*) should be evaluated by expert blood cell morphology.

2.5 Platelets

The number of platelets is related to the bleeding risks and therefore an important parameter for pre-operative screening and diagnosis. For microscopic platelet counting a counting chamber is used and platelets can be counted using the microscope phase contrast technique. This manual technique, however, is imprecise and time consuming especially when the platelet count is low[44]. The automated counting of platelets is now the rule rather than the exception, because automated platelet counting is precise and in a short time a lot of cells can be counted. However, spurious platelet counts are observed in pseudo thrombocytosis, and pseudo thrombocytopenia.
The use of anticoagulans can cause pseudo thrombocytopenia[45-47], which is not recognized by the automated platelet counting, while microscopic examination of the blood shows aggregated platelets. Another way of blood sampling is necessary to obtain a correct platelet count[48]. Also giant platelets may interfere with red blood cells and can therefore be counted as red blood cells and not as platelets[49].
A spurious thrombocytosis can be caused by the presence of pseudoplatelets[50], cryoglobulins[51] and bacteria[52]. Small vesicles mimicking platelets, originated from leukocytes, may appear when sepsis[50] or malignant disorders[53] are present. Acute leukaemias often are accompanied with haemorrhagic diathesis, which may be caused by thrombocytopenia[54]. In such patients, a spurious platelet count due to fragmentation of white blood cells has been reported[55-57]. Staining blood smears of a patient suffering from acute leukaemia with a normal platelet count, using platelet-specific antigen resulted in 4% positive cells, whereas one third part of the particles showed staining characteristics identical to the leukaemia cells. These results point to the leukaemic origin of the pseudoplatelets[55]. Recognition of pseudoplatelets in this group of patients is very important because of the risk of bleeding (*figure 6*).



Normal platelets *Pseudoplatelets* *Macrocytic platelets* *Agranular platelets*

Platelet aggregates *Platelet satellitism*

fig 6
Morphology of platelets

Cryoglobins may cause an erroneous platelet count because their size may be equal to platelets[51]. In these cases immuno platelet counting should be performed. When bacteria are present in small numbers in peripheral blood, a delay in the processing of the blood sample may cause increment in the number of bacteria. These bacteria also can interfere with platelet counting[52].
One should be aware that in many cases platelet sized particles could lead to a spurious elevated platelet count. Microscopic examination of the blood smear may track suspicious cases, and immuno platelet counting using anti-CD61 may be an alternative to report a correct platelet count[58].
As mentioned before presence of platelet-sized particles may lead to falsely elevated platelet counts. In spite of the fact that platelet counts are performed using impedance techniques or flow cytometry on a routine analyser these platelet-sized particles may be not recognized. Microscopic counting in a counting chamber is not an alternative since this method is also based on particle size. Observation of a stained blood smear is the only way to detect the so-called pseudoplatelets. Counting platelets using antibodies in combination with flow cytometry should lead to an accurate platelet count.

3 Alternative diagnostics for sepsis

Infection can lead to sepsis, which can ultimately cause septic shock. As mortality increases during the development of septic shock, early

detection of sepsis is very important. Infection and sepsis can be accompanied by various abnormalities in blood cells, both quantitatively and morphologically. A reaction on infection is an increase of the neutrophils (neutrophilia), or a decrease of neutrophils (neutropenia) due to consumption. Often, infection is accompanied by toxic granulation and/or vacuolisation of the white blood cells. Immature granulocytes may appear such as band cells and metamyelocytes. Because of the diagnostic and statistic limitations of cell morphology, alternative diagnostic tests have been developed[59-61].
Other than haematological parameters have been developed to diagnose infection or sepsis, such as the measurement of C-reactive protein (CRP). CRP has its limitations[62, 63], white blood cell differentiation is still extensively used for evaluation of infection or sepsis. Right now we are at a turning point with respect to sepsis diagnosis. Interleukins and cell surface markers gain ground and seem to be more specific. An important drawback of these tests, however, is the high price per test.

3.1 Immature granulocytes

Immature granulocytes appear as a result of the release of premature cells from the bone marrow, caused by cytokine stimulation, and may therefore be a sign for infection[64]. Compared to enumeration of band cells, counting of immature granulocytes is less controversial, and may be a good discriminator between infected and non-infected patients[65]. Using special developed software, the Sysmex XE-2100 haematology analyser is capable of counting immature granulocytes in an accurate and relative easy way[26]. Based on a combination of fluorescent staining and sideward scatter signals, immature granulocytes can be separated from mature granulocytes.

3.2 CD64

Apart from changes in neutrophils and their precursors, cell surface markers (CD markers) can change and might therefore be used as a marker for infection. Measurement of CD markers is labour intensive and complex. CD64 is a high affinity Fcγ membrane receptor, which binds monomeric as well as aggregated IgG. During the early stage of granulopoiesis CD64 is expressed. Mature granulocytes do not express this receptor[66]. While activated mature granulocytes show elevated levels of this membrane protein[67, 68].

3.3 Other sepsis parameters

Other parameters could contribute to the diagnosis of sepsis. For example interleukin-6 is associated with severity and outcome of meningococcal septicaemia[69]. Early medical complications may be predicted by interleukin-8 when measured at fever onset in patients with gram-negative bacteraemia[59]. However, the potential usefulness of cytokine measurements in combination with prognostic scores to identify patients who may benefit from treatment with anti-inflammatory or antithrombotic therapies should be further evaluated[60].

Another potential marker might be procalcitonin. Procalcitonin indicates not only a probable bacterial infection, but also progression of infection into sepsis or septic shock. Despite these findings there are no clinical studies available checking the outcome[61, 70].

The probable value and usefulness of the above mentioned parameters need to be evaluated.

This object is beyond the scope of this thesis.

4 The aim of this thesis

The aim of this thesis is to investigate the efficacy of microscopic and automated blood cell differentiation. Furthermore, to evaluate a new promising sepsis marker as alternative for band cells. Since better or alternative methods are avialable to obtain the same result compared to white blood cell morphology[71], some controversial morphology is investigated. Moreover, the important question, to maintain the microscopic differential, is answered.

References

1 Houwen B. The differential cell count. Lab Hematol 2001;7:89-100
2 Elghetany MT. Surface antigen changes during normal neutrophilic development: a critical review. Blood Cells Mol Dis 2002;28:260-274
3 Rümke CL. Imprecision of ratio-derived differential leukocyte counts. Blood Cells 1985;11:311-314
4 Lee GR, Foerster J, Lukens J et al. Wintrobe's Clinical Hematology. 10th ed. Baltimore, Williams & Wilkins. 1999
5 Lichtmann MA in Williams Hematology 4th ed. McGraw-Hill Publishing Company 1991
6 Torlakovic E, Hibbs JR, Miller JS, Litz CE. Intracellular bacteria in blood smears in patient with central venous catheters. Arch Intern Med 1995;155:1547-1550
7 Rivera P, Wilson M, Rubinfeld RS. Pneumococcal keratitis, bacteremia, and septic arthritis in an asplenic patient. Cornea 1996;15:434-436
8 Scott MA, Gobert-Jones J. Intracellular organisms in peripheral blood in a case of meningococcal septicaemia. Clin Lab Haemat 1990;12:477-478
9 Blot F, Nitenberg G, Chachaty E, Raynard B, Germann N, Antoun S, Laplanche A, Brun-Buisson C, Tancrède C. Diagnosis of catheter-related bacteraemia: a prospective comparison of the time to positivity of hub-blood versus peripheral-blood cultures. Lancet 1999;354:1071-1077
10 Press OW, Ramsey PG, Larson EB, Fefer A, Hickman RO. Hickman catheter infections in patients with malignancies. Medicine 1984;63:189-200
11 Kite P, Dobbins BM, Wilcox MH, McMahon MJ. Rapid diagnosis of central-venous-catheter-related bloodstream infection without catheter removal. Lancet 1999;354:1504-1507
12 Fife A, Hill D, Barton C, Burden P. Gram negative septicaemia diagnosed on peripheral blood smear appearances. J Clin Pathol 1994;47:82-84
13 Pedersen G, Schonheider HC, Nielsen LC. Capnocytophaga canimorsus bacteraemia demonstrated by a positive peripheral blood smear. APMIS 1993;101:572-574
14 Krause JR. Automated differentials in the hematology laboratory. Am J Pathol 1990;4:S11-S16
15 Tatsumi N, Pierre R. Automated image processing: past, present, and future of blood cell morphology identification. Clin Lab Med 2001;22:299-316
16 Buttarello M, Gadotti M, Lorenz C, Toffalori E, Ceschini N, Valentini A, Rizzotti P. Evaluation of four automated hematology analyzers. A comparative study of differential counts (imprecision and inaccuracy). Am J Clin Pathol 1992;97:345-352
17 Warner BA, Reardon DM, Marshall DP. Automated haematology analysers: a four-way comparison. Med Lab Sci 1990;47:285-296
18 Swaim WR. Laboratory and clinical evaluation of white blood cell differential counts. Comparison of the Coulter VCS, Technicon H-1, and 800-cell manual method. Am J Clin Pathol 1991;95:381-388
19 Corberand JX, Laharrague PF, Fillola G, Charlet JP, Cambus JP. Discovery of unsuspected pathological states using a new haematology analyser. Med Lab Sci 1991;48:80-83
20 Wersch van JW, Bank C. A new development in haematological cell counting: the Sysmex NE-8000, automaton for cell count and physical five-part leukocyte differentiation. J Clin Chem Clin Biochem 1990;28:233-240

21 De Keijzer MH, van der Meer W. Automated counting of nucleated red blood cells in blood samples of new-borns. J Clin Lab Haematol 2002;6:343-346
22 Stamminger G, Auch D, Diem H, Sinha P. Performance of the XE-2100 leucocyte differential. Clin Lab Haematol 2002;24:271-280
23 Jones RG, Faust A, Glazier J, Matthews R, Potter B. CELL-DYN 4000: Utility within the core laboratory structure and preliminary comparison of its expanded differential with the 400-cell manual differential count. Lab Hemat 1998;4:34-44
24 Siekmeier R, Bierlich A, Jaross W. The white blood cell differential: three methods compared. Clin Chem Lab Med. 2001;39(5):432-445
25 Koenn ME, Kirby BA, Cook LL, Hare JL, Hall SH, Barry PM, Hissam CL, Wojcicky SB. Comparison of four automated hematology analyzers. Clin Lab Sci 2001 Fall;14(4):238-242
26 Ansari-Lari MA, Kickler TS, Borowitz MJ. Immature granulocyte measurement using the Sysmex XE-2100 : relationship to infection and sepsis. Am J Clin Pathol 2003;120:795-799
27 Briggs C, Kunka S, Fujimoto H, Hamaguchi Y, Davis BH, Machin SJ. Evaluation of immature granulocyte counts by the XE-IG master: upgraded software for the XE-2100 automated hematology analyzer. Lab Hematol 2003;9:117-124
28 Abe Y, Wada H, Sakakura M, Nishioka J, Tomatsu H, Hamaguchi Y, Oguni S, Shiku H, Nobori T. Usefulness of fully automated measurement of reticulated platelets using whole blood. Clin Appl Thromb Hemost 2005;11:263-270
29 Thomas L, Franck S, Messinger M, Linssen J, Thome M, Thomas C. Reticulocyte hemoglobin measurement - comparison of two methods in the diagnosis of iron-restricted erythropoiesis. Clin Chem Lab Med 2005;43:1193-1202
30 Molero T, Roemer B, Perera Alvarez Mdel M, Lemes A, Inigo Sde L, Palacios G, Scott CS. Analysis and enumeration of T cells, B cells and NK cells using the monoclonal antibody fluorescence capability of a routine haematology analyser (Cell-Dyn CD4000). Clin Lab Haematol 2005;27:224-234
31 Little BH, Robson R, Roemer B, Scott CS. Analysis and enumeration of T cells, B cells and NK cells using the monoclonal antibody fluorescence capability of a routine haematology analyser (Cell-Dyn CD4000). Clin Lab Haematol 2005;27:224-234
32 Van der Meer W, Dinnissen JWB, de Keijzer MH, Scott CS. Comparative evaluation of Abbott Cell-Dyn CD3700, Bayer ADVIA 120 and Sysmex XE-2100 automated leucocyte differentials and flagging. Lab Haematol 2002;8:102
33 Seebach, JD, Morant R, Rüegg R, Seifert B, Fehr J. The diagnostic value of the neutrophil left shift in predicting inflammatory and infectious disease. Am J Clin Pathol 1997;107:582-591
34 Miale J. Laboratory Medicine: Hematology 6ed. St Louis, CV Mosby 1982
35 Glassy E. Color Atlas of Hematology: Illustrated Field Guide Based on Proficiency Testing. Northfield, IL, College of American Pathologists, 1998
36 Lee GR, Foerster J, Lukens J et al. Wintrobe's Clinical Hematology. 10th ed. Baltimore, Williams & Wilkins 1999
37 Cornbleet PJ. Clinical Utility of the band cell. Clin Lab Med 2002;22:101-136
38 Heagy W, Nieman K, Hansen C, Cohen M, Danielson D, & West MA. Lower levels of whole blood LPS-stimulated cytokinee release are associated with poorer clinical outcomes in surgical ICU patients. Surg Infect 2003;4:171-180
39 Saulsbury FT. B cell proliferation in acute infectious lymphocytosis. Pediatr Infect Dis J 1987;6:1127-1129

40 Hutchinson RE, Kurec AS, Davey FR. Lyphocytic surfaces markers in lymphoid leukomoid reactions. Clin Lab Med 1988;8:237-245
41 Leroy JG, De Mars RI; Mutant enzymatic and cytological phenotypes in cultured human fibroblasts. Science 1967;157:804-806
42 Okada S, Owada M, Sakiyama T, Yutaka T, Ogawa M. I-cell disease: clinical studies of 21 Japanese cases. Clin Gen 1985;28:207-215
43 Kamiya M, Tada T, Kuhara H et al. I-cell disease A case report and review of the literature. Acta Pathol Jpn 1986;36:1679-1692
44 Bain BJ; Blood cells: A practical guide. 3d ed. Blackwell Sciences, Oxford, 2002
45 Lombarts AJ, Zijlstra JJ, Peters RH, Thomasson CG, Franck PF. Accurate platelet counting in an insidious case of pseudothrombocytopenia. Clin Chem Lab Med 1999;11-12:1063-1066
46 Casonato A, Bertomoro A, Pontara E, Dannhauser D, Lazzaro AR, Girolami. EDTA dependent pseudothrombocytopenia caused by antibodies against the cytoadhesive receptor of platelet gpIIB-IIIA. J Clin Pathol 1994;47:625-630
47 Bizzaro N. Platelet satellitosis to polymorphonuclears: cytochemical, immunological, and ultrastructural characterization of eight cases. Am J Hematol. 1991;36:235-242
48 van der Meer W, Allebes W, Simon A, van Berkel Y, de Keijzer MH. Pseudothrombocytopenia: report of a new method to count thrombocytes in a patient with EDTA and temperature independent antibodies of the IgM type. Eur J Haematol 2002;69:243-247
49 de Keijzer MH, Swinkels DW, van der Meer W. Thrombocytopenia and giant platelets without major hemorrhagic complications in a pregnant patient. Ann Clin Biochem 1998;35:678-680
50 Hanker JS, Giamarra BL. Neutrophil pseudoplatelets: Their discrimination by myeloperoxidase demonstration. Science 1983;220:415-417
51 Ahsen N von, Ehrlich B, Scott CS, Riggert J, Oellerich M. Cryoglobulins interferre with platelet counts by optical and impedance methods but not with the CD61 immunoplatelet count. Clin Chem 2001;10:1858-1860
52 Kakkar N. Spurious rise in the automated platelet count because of bacteria. J Clin Pathol 2004;57:1096-1097
53 Malcolm ID, Monks P, Katz M. Spurious thrombocytosis in acute myelocytic leukemia. N Engl J Med 1978;298:1260-1261
54 Wilde JT, Davies JM. Haemostatic problems in acute leukaemia. Blood Rev 1990;4:245-251
55 Hammerstrom J. Spurious platelet counts in acute leukaemia with DIC due to cell fragmentation. Clin Lab Haemat 1992;14:239-243
56 Sugimoto T, Saigo K, Ryo R. Giant platelet-like cell fragments produced from abnormal promyelocytes in acute myelogenous leukemia. Rinsho Byori 1998;46:182-185
57 Li S, Salhany KE. Spurious elevation of automated platelet counts in secondary acute monocytic leukemia associated with tumor lysis syndrome. Arch Pathol Lab Med 1999;11:1111-1114
58 Norris S, Pantelidou D, Smith D, Murphy MF. Immunoplatelret counting: potential for reducing the use of platelet transfusions through more accurate platelet counting. Brit J Haematol 2003;121: 605-613
59 Engel A, Knoll S, Kern P, Kern WV. Interleukin-8 serum levels at fever onset in patients with neutropenia predict early medical complications. Infection 2005;33:380-382

60 Oberholzer A, Souza SM, Tschoeke SK, Oberholzer C, Abouhamze A, Pribble JP, Moldawer LL. Plasma cytokine measurements augment prognostic scores as indicators of outcome in patients with severe sepsis. Shock. 2005;23:488-93
61 Meisner M. Biomarkers of sepsis: clinically useful? Curr Opin Crit Care 2005;11:473-480
62 Manucha V, Rusia U, Sikka M, Faridi MM, Madan N. Utility of haematological parameters and C-reactive protein in the detection of neonatal sepsis. J Paediatr Child Health 2002;38:459-464
63 Aikawa N, Fujishima S, Endo S, Sekine I, Kogawa K, Yamamoto Y, Kushimoto S, Yukioka H, Kato N, Totsuka K, Kikuchi K, Ikeda T, Ikeda K, Harada K, Satomura S. Multicenter prospective study of procalcitonin as an indicator of sepsis. J Infect Chemother. 2005;11:152-159
64 Selig C, Nothdurft W. Cyokines and progenitor cells of granulopoiesis in peripheral blood of patients with bacterial infections. Infect Immun 1995;63:104-109
65 Ardon MJ, Westengard JC, Dutcher TF. Band neutrophil counts are unnecessary for the diagnosis of infection in patients with normal total leukocyte counts. Am J Clin Pathol 1994;102:646-649
66 Elghetany MT, Ge Y, Patel J, Martinez J, Uhrova H. Flow cytomtric study of neutrophilic granulopoiesis in normal bone marrow using an expanded panel of antibodies: correlation with morphologic assessments. J Clin Lab Anal 2004; 18:36-41
67 Allen E, Bakke AC, Purtzer MZ, Deodhar A. Neutrophil CD64 expression: Distinguishing acute inflammatory autoimmune disease from systemic infections. Am Rheum Dis 2002;61:522-525
68 Davis BH, Bigelow NC, Curnutte JT, Ornvold K. Neutrophil CD64 expression. Potential diagnostic indicator of acute inflammation and therapeutic monitor of interferon-γ therapy. Lab Hematol 1995;1:3-12
69 Pathan N, Williams EJ, Oragui EE, Stephens AC, Levin M. Changes in the interleukin-6/soluble interleukin-6 receptor axis in meningococcal septic shock. Crit Care Med 2005;33:1839-1844
70 Clech C, Ferriere F, Karoubi P.*et al*. Diagnostic and prognostic value of procalcitonin in patients with septic shock. Crit Care Med 2004; 32:1166-1169
71 Houwen B. White blood cell morphology in the balance. Lab Hematol 2005;11:79-82

Chapter 2

Bacteria in blood smears: overwhelming sepsis or trivial contamination

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Abstract
It is unusual to find micro-organisms in peripheral blood smears, and their presence is frequently associated with overwhelming sepsis and consequently a poor prognosis. In this report we demonstrate four cases with bacteria in blood smears. Two of them resulted in a fatal outcome, but the other two were caused by a contamination either via the central venous catheter or *in vitro*, both without dramatic outcome. The finding of bacteria in blood smears has to be interpreted carefully and thorough examination of peripheral blood smears may be of great importance in the early diagnosis of bacteremia. However, *in vitro* contamination must be excluded.

Introduction

Generally the presence of bacteria in blood is demonstrated by conventional blood cultures. Buffy coat smears have proved to be a fast method for detecting intracellular bacteria in blood[1-5]. Sometimes bacteria are seen in routinely obtained blood smears and become sufficiently numerous to be identified in the terminal phase of an overwhelming sepsis[6-8].
In the specific case of *Capnocytophaga canimorsus* septicaemia, bacteria are present in large numbers in the circulation in the early stage of the sepsis, and can therefore be seen in peripheral blood smears[9, 10]. Immediate suspicion of the infection may lead to a prompt and adequate treatment[11]. The result of the blood culture for *Capnocytophaga canimorsus*, on the other hand, is only available after several days[10, 12].
The finding of intracellular bacteria in routine blood smears prepared from a blood sample taken from a central venous catheter (CVC) most likely indicates catheter related infection[6, 13, 14]. It is then important to remove the CVC because bloodstream infection is associated with a significant degree of morbidity: case mortality of catheter-related bloodstream infection is 10%-20%[15].
Finally, blood samples obtained by finger or heel prick may contain bacteria or fungi as a consequence of *in vitro* contamination. In these situations bacteria will only be present extracellularly, although, if a long period has elapsed from the time of the puncture to the moment of preparing the blood smear, phagocytosis by neutrophils may lead to the bacteria being intracellular.
Thorough investigation of the smears may result in the identification of a severe sepsis, a CVC related infection or an *in vitro* contamination. In this report we describe that importance of the microscopic detection of intra- and extracellular bacteria in blood smears. Four cases are

presented of blood smears of patients with severe sepsis with infected catheters or with *in vitro* contamination.

Case 1

A six-month-old baby was admitted to the Emergency Department with high fever and suspicion of septic shock. There were no clinical signs of meningitis. Respiratory insufficiency was present and the patient was intubated, and a CVC was inserted. The patient was transported to the paediatric intensive care unit, where therapy with ceftriaxon and dexamethason was started. Subsequently inotropics were given. The patient developed a bradycardia followed by cardiopulmonary resuscitation, and died the same day.

The routine laboratory tests showed no remarkable abnormalities with the exception of a blood ammonia concentration of 279 μmol/L (reference value for our laboratory: < 60 μmol/L), and some abnormal coagulation parameters: thromboplastin 100 sec, activated partial thrombo plastin time (APTT) 300 sec and fibrinogen 0.110 g/L (reference values for our laboratory: 14-17 sec, 20-38 sec and 1.6 – 3.2 g/L respectively). The haemoglobin concentration decreased from 5.6 mmol/L to 2.2 mmol/L and the platelet count decreased during the same period from 47 x 10^9/L to < 10 x 10^9/L. Blood smears were stained according to the May-Grünwald Giemsa method. No neutrophils were seen in the blood smears; however, on autopsy it was found that the lungs were massively infiltrated with neutrophils. Post mortem samples of the various tissues were cultured; no bacteria were found. In the routinely blood smears diplococcus-like bacteria were seen which were almost always adhered extracellularly to the erythrocytes (*figure 1*).

Case 2

A previously healthy, 46-year-old man was referred to the hospital because of hypotension and abdominal pain. Medical history revealed a splenectomy due to trauma 19 years before. At physical examination there was dyspnoe, low blood pressure and high fever.

The abdomen was not distended and showed a left upper quadrant incision; on the patient's left hand there were several small, 3-4 mm deep, defects and one lesion of 2 cm with a black non-infected rim due to a dog bite, three days before. The white blood cell count was 3.8 x 10^9/L, platelet count was 68 x 10^9/L, APTT 69 sec, prothrombin time (PT) 1.9 INR and the C-reactive protein (CRP) was 150 mg/L (reference value for our laboratory: <10 mg/L). Other routine laboratory tests were normal.

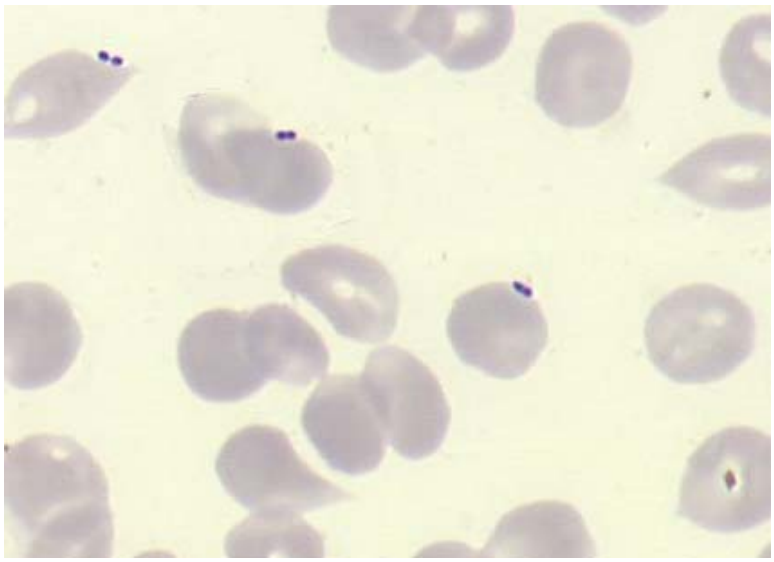

fig 1
Blood smear of patient 1 with bacteria which are adhered at erythrocytes (900x)

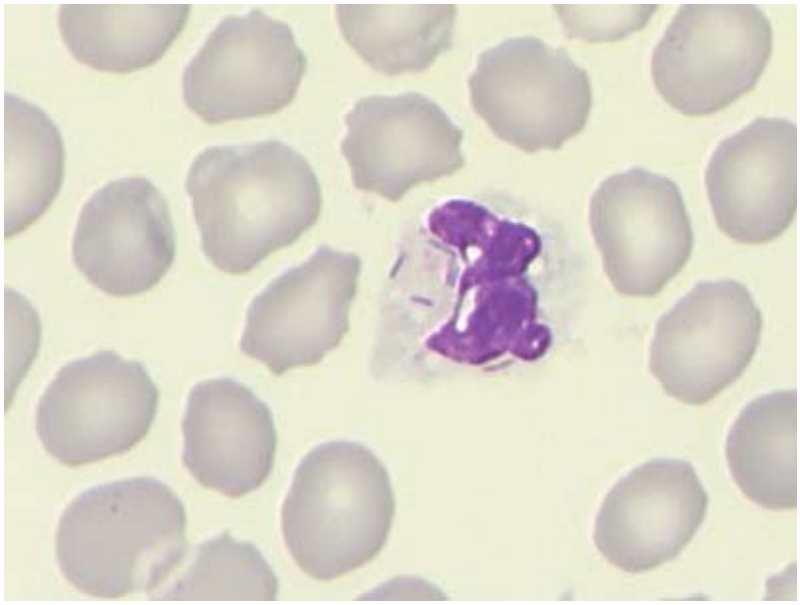

fig 2
Blood smear of patient 2 with intracellular presence of Capnocytophaga Canimorsus (900x)

The blood smears were stained according to the May-Grünwald Giemsa method. The differentiation of the leukocytes seemed to be normal. However, in the neutrophils some rod shaped inclusions were seen (*figure 2*). Three days later blood cultures showed that the blood was infected with *Capnocytophaga canimorsus*, and the patient received amoxicillin-clavulanic acid. Treatment with high doses of inotropics, artificial respiration, transfusions and hemodialysis was necessary. Despite prolonged and adapted antibiotic therapy, the patient developed pancreatic necrosis, pulmonary infection (*Pseudomonas*) and necrosis of the left forearm. The patient died on day 21 due to multi-organ failure.

Case 3

A three-year-old girl was admitted to the hospital because of pneumatosis intestinalis (PI). One year before she had been diagnosed with neuroblastoma stage IV. At the present admission she had abdominal pain and bloody diarrhoea. Physical examination showed a cheerful girl who did not seem to be ill. The exit site of her CVC was a little inflamed. Abdominal examination did not reveal signs of peritonitis. Laboratory investigations showed thrombocytopenia with a platelet count of 53 x 10^9/L without neutropenia; the CRP was < 10 mg/L. X-ray of the abdomen showed PI of the colon ascendens, flexura hepatica, and mesocolon. Furthermore, little free air was seen beneath the diaphragm. Because of the pneumatosis, intravenous antibiotics were started prophylactically (ceftazidime, metronidazol). After four days the patient developed fever and her clinical condition worsened. A blood culture sample, drawn from the CVC, yielded a *Staphylococcus epidermis*, and a second culture of a sample taken from the CVC two days later, *Staphylococcus aureus*. Nine days after admission the CVC was removed, and the child recovered.

From the fourth day until the ninth day of admission, blood smears drawn from the CVC showed intracellular and extracellular bacteria (*figure 3*). A peripheral blood smear taken by finger puncture on the sixth day was negative.

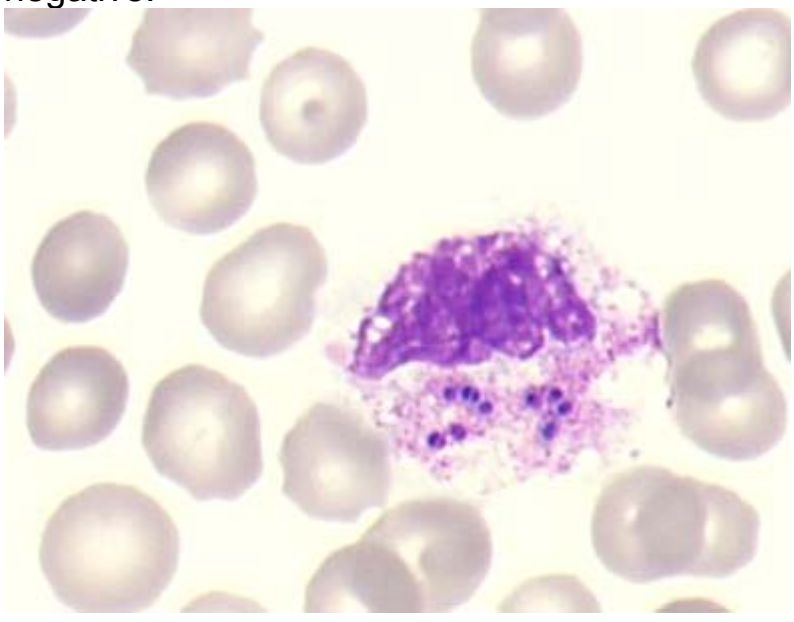

fig 3

Blood smear of patient 3 with intracellular cocci in pairs (900x)

Case 4

An eight-year-old girl had been treated for acute lymphoblastic leukaemia. The patient stayed in remission and was seen for routine check-up. In the blood smear bacteria, mainly extracellular, were found (*figure 4*), whereas the blood cell count appeared to be normal.

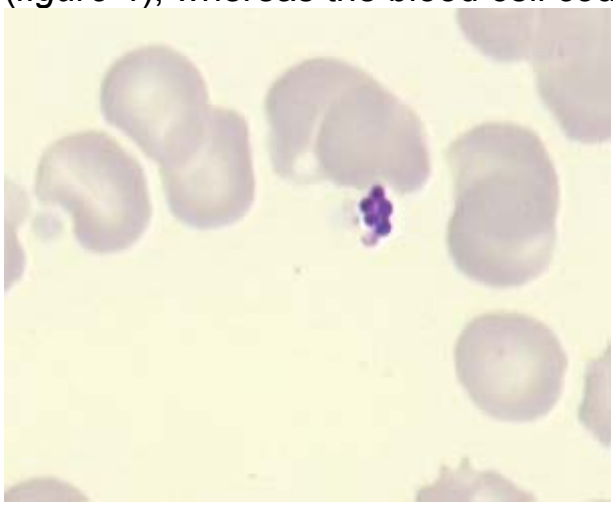

fig 4
Blood smear of patient 4 with extracellular bundle of cocci (900x)

The patient had no fever nor other indications that she was developing a sepsis. A second investigation of the blood smear showed both extracellular bacteria and some undefined coloured pieces filled with bacteria (*figure 5*). No abnormalities were seen at the next visit.

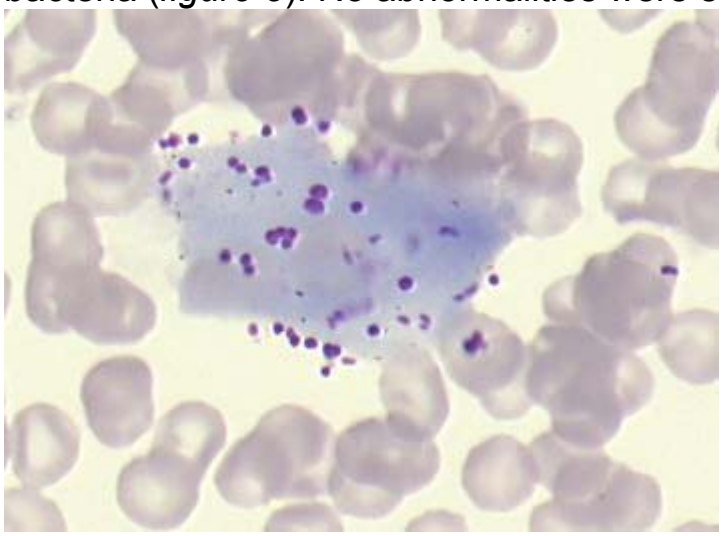

fig 5
Blood smear of patient 4 with undefined coloured piece filled with similar bacteria as in Fig 4 (600x)

Discussion

Although bacteria could not be cultured in case 1, the combination of the clinical features and the appearance of the blood smears, makes it likely

that the patient died due to an infection with *Streptococcus pneumoniae*. It is known that *Streptococcus pneumoniae* can bind on epithelial cells and a mutant has been identified which can attach to all cell types[16, 17]. The morphological characteristics of the bacteria, which adhered to erythrocytes, are similar to those of *Streptococcus pneumoniae*.

The risks of overwhelming sepsis in asplenic patients has been recognised over the past decades[7, 18]. Several authors reported an infection with *Capnocytophaga canimorsus*, especially in patients who had undergone splenectomy[9-12, 18, 19]. The finding of these rod-shaped bacteria in case 2 was the first indication for the infection with this bacteria, and was indicative for a bad prognosis. In these patients immediate treatment should be desirable.

Intracellular or extracellular bacteria are frequently found in blood smears from patients with central venous catheters (CVC)[6, 13-15, 20]. In most cases the patient has an underlying neoplasm and is under treatment with immunosuppressive medication. In case 3 blood puncture was repeated after seeing bacteria in the blood smears. Again bacteria were found. Morphologically these bacteria resembled *Staphylococcus*. Cocci were seen both intracellularly in neutrophils and extracellularly, most frequently in pairs. Blood smears obtained by finger puncture did not contain bacteria. Blood drawn from the CVC was cultured for bacteria, and *Staphylococcus epidermis* was found. Once the catheter was removed no bacteria were seen in subsequent blood smears drawn by vene puncture. When bacteria are seen in blood smears drawn by CVC, we recommend that a finger puncture should be done to distinguish catheter-related sepsis.

Finally, we described a case with bacteria in a blood smear of a patient without any signs or symptoms of infection. Both intracellular and extracellular bacteria were present, mostly in small bundles. In addition, some undefined coloured pieces were seen in the blood smear filled with similar bacteria. This led us to the conclusion *in vitro* contamination.

The presence of bacteria in peripheral blood smears may point to bacteremia[2,10]. For this reason it is important to report the presence of bacteria. However, spurious bacterial findings in blood may be due to contamination of the anticoagulant, glassware and so on. For further identification a Gram stain can be used. When intracellular bacteria are detected, an *in vivo* infection should be suspected as long as the blood smear is prepared using a fresh blood sample. To distinguish bacteremia and *in vitro* contamination a new blood sample should be obtained. The presence of bacteria in blood smears drawn by CVC in combination with the absence of bacteria in blood drawn by finger puncture is likely to indicate catheter-related sepsis, which may necessitate removal of the catheter.

References

1 Reik H, Rubin SJ. Evaluation of the buffy coat smear for rapid detection of bacteremia. JAMA 1981;245:357-359
2 Brooks GE, Pribble AH, Beaty HN. Early diagnosis of bacteremia by buffy coat examination. Arch Intern Med 1973;132:673-675
3 Fife A, Hill D, Barton C, Burden P. Gram negative septicaemia diagnosed on peripheral blood smear appearances. J Clin Pathol 1994;47:82-84
4 Humphrey AA. Use of the buffy layer in the rapid diagnosis of septicemia. Am J Clin Pathol 1944;14:358-362
5 Ristuccia PA, Hoeffner RA, Digamon-Beltran M, Cunha BA. Detection of bacteremia by buffy coat smears. Scan J Infect Dis 1987;19:215-217
6 Torlakovic E, Hibbs JR, Miller JS, Litz CE. Intracellular bacteria in blood smears in patient with central venous catheters. Arch Intern Med 1995;155:1547-1550
7 Rivera P, Wilson M, Rubinfeld RS. Pneumococcal keratitis, bacteremia, and septic arthritis in an asplenic patient. Cornea 1996;15:434-436
8 Scott MA, Gobert-Jones J. Intracellular organisms in peripheral blood in a case of meningococcal septicaemia. Clin Lab Haemat 1990;12:477-478
9 Pedersen G, Schonheider HC, Nielsen LC. Capnocytophaga canimorsus bacteraemia demonstrated by a positive peripheral blood smear. APMIS 1993;101:572-574
10 Rubin SJ. DF-2: A fastidious fermentative Gram-negative rod. Eur J Clin Microbiol 1984;3:253-257
11 Kristensen KS, Winterheik M, Rasmussen ML. Capnocytophaga canimorsus infection after dog bite. Lancet 1991;337:849
12 Kalb R, Kaplan MH, Tenenbaum MJ, Joachim GR, Samuels S. Cutaneous infection at dog bite wounds associated with fulminant DF-2 septicemia. Am J Med 1985;78:687-690
13 Blot F, Nitenberg G, Chachaty E, Raynard B, Germann N, Antoun S, Laplanche A, Brun-Buisson C, Tancrède C. Diagnosis of catheter-related bacteraemia: a prospective comparison of the time to positivity of hub-blood versus peripheral-blood cultures. Lancet 1999;354:1071-1077
14 Press OW, Ramsey PG, Larson EB, Fefer A, Hickman RO. Hickman catheter infections in patients with malignancies. Medicine 1984;63:189-200
15 Kite P, Dobbins BM, Wilcox MH, McMahon MJ. Rapid diagnosis of central-venous-catheter-related bloodstream infection without catheter removal. Lancet 1999;354:1504-1507
16 Cundell DR, Gerard NP, Gerard C, Idanpaan-Heikkila T, Tuomanen EI. Streptococcus pneumoniae anchor to activated human cells by the receptor for platelet-activating factor. Nature 1995;377:435-438
17 Spellerberg B, Cundell DR, Sandros J, Pearce BJ, Idanpaan-Heikkila I, Rosenow C, Masure HR. Pyruvate oxidase, as a determinant of virulence in Streptococcus Pneumoniae. Mol Biol 1996;19:803-813
18 Styrt B. Infection associated with asplenia: risks, mechanisms, and prevention. Am J Med 1990;88:33N-42N
19 Martone WJ, Zuehl RW, Minson GE, Scheld WM. Postsplenectomy sepsis with DF-2: Report of a case with isolation of the organism from the patient's dog. Ann Intern Med 1980;93:457-458
20 Raad I. Intravascular-catheter-related infections. Lancet 1998;351:893-898

Chapter 3

Automated flagging influencing the inconsistency and bias of band cell and atypical lymphocyte morphological differentials

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Abstract

This study evaluated inter- and intra-observer variability of band cell and atypical lymphocyte differentials and the influence of instrument flagging information on resulting microscopic differentials. Five stained slides with a range of band cell counts and five with variable numbers of atypical lymphocytes were sent for morphological review by 30 technicians. No supplementary full blood cell count information was provided. Two months later, the same slides were sent together with their corresponding analyser reports comprising the full blood cell count, automated differentials and flags to the same technicians. The first and second appraisals of band cells and variant lymphocytes both showed poor levels of inter-observer consistency. Observed values for all slides were very wide and suggested a high inherent predisposition to erroneous reporting practices. Analysis of category trends showed that analyser left shift or IG flags had no influence on observer band cell assessments as downward versus upward category revisions were evenly balanced. The findings for atypical lymphocytes were, however, somewhat different. Two slides with no flags both showed balanced category revisions whereas two of the three slides with atypical lymphocyte flags showed clear evidence of upward category revision. The third slide with an atypical lymphocyte flag did not show any overall category trend but six of the seven observers who in the first examination recorded atypical lymphocyte estimates of $\leq$30% revised their estimates upwards when the slides were examined the second time. These results suggest that morphologist access to an analyser report and flagging information is unlikely to affect the 'randomness' of band cell determinations but it may induce observer bias in variant lymphocyte estimates.

Introduction

The leukocyte differential count is an important component of the routine full blood count[1]. A conventional microscopic differential, which is typically obtained by visual classification of 100 leucocytes as percentages of the five primary cell populations, is however labour-intensive and has poor confidence limits related to the inherent influence of Poisson statistics[2]. In addition, morphological differentials often consider qualitative morphological features such as atypical granularity, nuclear shape and cytoplasmic colouring and further attempts to quantify atypical elements such as band cells (non-segmented neutrophils) and 'variant' lymphocytes[3, 4]. While this additional information may have some weight in clinical decision-making processes, there are a number of important limitations to this particular aspect of morphological

appraisal that need to be considered. For example, although the descriptive definition of a band cell is well documented[5-7], the ability of individuals to consistently interpret and apply these descriptive guidelines is actually quite poor[8]. This problem is even more relevant to 'variant' lymphocytes where there are no standardised definitions and it is left to the morphologist to subjectively decide whether any given cell is normal or atypical.

The introduction of cell counters able to generate automated five-part differentials (as both relative and absolute counts) resolved many of the statistical limitations of the microscopic differential[9-12]. Moreover, the general reliability of automated leukocyte differentials has significantly reduced conventional morphological reviews. Nevertheless, there remains a requirement for morphologists to assess and quantify abnormal cell components in certain situations. As one of the facilitating 'triggers' for formal film examination, laboratories commonly utilise analyser 'flags' or alerts for the suspected presence of abnormal leucocytes. However, the possibility that these may be relatively inefficient is not well understood, a point that is illustrated by a recent report[13] that noted false positive flagging of almost 10% of haematologically normal samples as well as a failure to flag 14% of abnormal samples. The limitations of some analyser flags may thus lead to further inconsistencies in any subsequent morphological classification of abnormal cell types. In this context, especially where morphological distinctions are marginal, it is feasible that individual morphologists could be unduly influenced or biased by analyser flags for abnormal cells when morphological review is undertaken. In examining this hypothesis, this study evaluated the inter- and intra-observer variability of band cell and atypical lymphocyte differentials, and further assessed the influence of instrument flagging information on the resulting microscopic differential.

Materials and methods

Samples Studied:

In this study, five patient samples (A1 to A5) with a range of band cell counts and five samples with variable numbers of atypical lymphocytes (B1 to B5) were selected by the Nijmegen coordinating laboratory. As initial reference, two experienced technicians each differentiated 200 cells (*table 1*). For band cells, the morphological criteria of the Netherlands Society for Laboratory Haematology were used[7] which defines band cells as neutrophils with a nuclear constriction width of one half to one third of the maximum nuclear width. For atypical lymphocytes,

table 1 Abnormal cell characteristics and influence of flagging information on morphological assessments[a].

A: Neutrophil precursors	Selection Differential[b]	Supplementary Flagging Information for Second Examination[c]	Category change[d]		
			Downwards	Unchanged	Upwards
A1	3% band cells	Left shift + IG +	0	30	0
A2	3% band cells	Left shift +	4	23	0
A3	15% band cells	IG +	12	9	9
A4	25% band cells 3% immature granulocytes	Left shift +	8	15	7
A5	20% band cells	IG +	8	12	10
B: Atypical Lymphocytes	**Selection Differential[b]**	**Supplementary Flagging Information for Second Examination[c]**	**Category change**		
			Downwards	**Unchanged**	**Upwards**
B1	90% lymphocytes <10% atypical lymphocytes	Atyps ++ LUC 6%	5	10	15
B2	45% lymphocytes <10% atypical lymphocytes	No flags LUC <5%	9	11	10
B3	75% lymphocytes 30% atypical lymphocytes	Atyps ++ LUC 9%	5	15	10
B4	65% lymphocytes 15% atypical lymphocytes	No flags LUC 8%	9	12	9
B5	95% lymphocytes 75% atypical lymphocytes	Atyps ++ LUC 28%	9	12	9

[a] Five coded slides (A1 to A5) with variable degrees of abnormality with granulocyte precursors, and five slides associated with atypical lymphoid cells (B1 to B5) were examined on two separate occasions by 30 haematology technicians. On the second occasion, the slides were accompanied by a full analyser report (Bayer Advia 120) containing WBC count, differential and flagging information.
[b] Selection differential obtained by two independent observers each recording 200 leucocytes. The percentage lymphocytes refers to the percentage of the complete differential and the percentage atypical lymphocytes corresponds to the percentage of the lymphocyte fraction with atypical morphology.
[c] Supplementary information provided in the form of a full analyser report. Flagging information shown here corresponds to those of most relevance. IG corresponds to immature granulocytes, Atyps to atypical lymphocytes, and LUC to large unstained cells.
[d] Category change indicates the number of downward, unchanged or upward category revisions (of 30 in total) when the second examination was compared with the first (*tables 2 and 3*). The four band cell categories used in this study were (i) 0-5%, (ii) 6-10%, (iii) 11-20% and (iv) >20%. The four variant lymphocyte categories used in this study were (i) 0-10%, (ii) 11-30%, (iii) 31-60% and (iv) >60%.

broader definitions of irregular cytoplasmic or nuclear shape were applied. The normal reference range of band cells in adult blood is generally regarded as $\leq$5% while the normal frequency of atypical

lymphocytes (expressed as a proportion of the lymphocyte fraction) is considered as ≤10%.
For each sample, a set of May-Grünwald Giemsa stained slides was prepared and sent for morphological review by 30 technicians based in three different laboratories. No supplementary full blood cell count (FBC) information was provided and the technicians were asked to independently perform a 100-cell leukocyte differential including proportionate determinations of band cells, immature granulocytes, atypical lymphocytes and blast cells. This initial assessment (First Examination) was followed two months later by circulation of the same slide sets (with different coding) accompanied by full automated analyser reports (Bayer Advia 120, Bayer, Tarrytown, USA) comprising the FBC, automated differentials and flags for atypical lymphocytes (Atyps), blasts, left shift and/or immature granulocytes (IG). This particular report format was selected as all 30 technicians involved in the morphological study had direct experience of working with this analyser.
Statistical Analysis:
Analysis of observer variability of band cell and atypical lymphocyte differential estimates was determined separately for both the First and Second Examinations. Paired comparisons further provided an assessment of intra-observer consistency and the influence of supplementary flagging information on possible observer bias. Inter-observer reproducibility was established by assessing variability (median and observed ranges) for each data set. Paired comparisons (First versus Second Examination) for each slide were then evaluated with the non-parametric Wilcoxon Signed Ranks Test and the Weighted Kappa test for category agreement.

Results

Summarised results for the series of five samples (A1 to A5) used for evaluating band cell variability are shown in table 2. The range of observed values for both the First and Second Examination was wide for all samples with the exception of slide A1 where the band cell count was low. Interestingly, a second slide in this series (A2) with low band cells showed much wider inter-observer variability. Of additional relevance was the finding that for the other three samples where most observers correctly recorded increased band cells, there were some morphologists whose assessments suggested normality. Paired comparisons (Wilcoxon Signed Ranks Test) between the First and Second Examinations however indicated no significant difference between the median band cell values (*table 2*). Compared to the inconsistency of band cell estimates, it is pertinent to note that the small percentage of immature

granulocytes (myelocytes and metamyelocytes) in slide A4 was recorded by virtually all (27/30) observers (data not shown). Moreover, the intra-observer paired comparisons of these immature granulocytes were all within statistically predicted 95% confidence limits[14].

table 2 Analysis of paired band cell and variant lymphocyte evaluation data.

Slide		First examination	Second examination	Difference[1]
A1	Mean (SD)	1.0%(1.2)	1.2%(1.0)	ns
	Observed range	0 – 5%	0 – 4%	
A2	Mean (SD)	2.8%(3.2)	2.4%(2.2)	ns
	Observed range	0 – 15%	0 – 8%	
A3	Mean (SD)	9.0%(5.5)	8.3%(5.7)	ns
	Observed range	2 – 27%	2 – 20%	
A4	Mean (SD)	19.0%(7.9)	19.2%(7.4)	ns
	Observed range	5 – 35%	7 – 40%	
A5	Mean (SD)	11.0%(6.6)	13.4%(7.5)	ns
	Observed range	1-24%	4-33%	
Slide				
B1	Mean (SD)	15.2%(17.0)	33.2%(15.2)	p=0.03
	Observed range	0 – 50%	5 – 100%	
B2	Mean (SD)	34.3%(28.4)	38.5%(29.4)	ns
	Observed range	0 – 100%	0 – 95%	
B3	Mean (SD)	15.9%(16.6)	23.3%(23.5)	ns
	Observed range	0 – 60%	0 – 100%	
B4	Mean (SD)	28.5%(28.5)	29.8%(32.0)	ns
	Observed range	0 – 100%	0 – 100%	
B5	Mean (SD)	58.0%(28.6)	61.7%(25.0)	ns
	Observed range	5 – 100%	0 – 100%	

[1] Wilcoxon Signed Ranks Test (2-tailed p) for differences between medians of related sample sets, ns indicates no significant difference (p>0.05).

Similar assessments of atypical lymphocytes revealed an extremely poor degree of observer consistency, although comparisons of First and Second Examinations did not indicate, with the exception of slide B1, any significant differences in the median values distributions. However, the interpretative relevance of this is minimal because of their wide observed ranges, and the limitations of paired data set comparisons when intra-observer trends (i.e. Second versus First Examination) are random.

To better understand and quantify this, the individual observer results were categorised into four groups according to reported severity of the abnormality. For band cells, these categories were 0-5%, 6-10%,

table 3 Intra-observer agreement for first and second band cell examinations.

Slide A1	*First reading*				
Second reading	0-5%	6-10%	11-20%	>20%	***Total***
0-5%	30	0	0	0	**30**
6-10%	0	0	0	0	**0**
11-20%	0	0	0	0	**0**
>20%	0	0	0	0	**0**
Total	**30**	**0**	**0**	**0**	**30**

Weighted Kappa test for category agreement: 1.00

Slide A2	*First reading*				
Second reading	0-5%	6-10%	11-20%	>20%	***Total***
0-5%	23	3	1	0	**27**
6-10%	3	0	0	0	**3**
11-20%	0	0	0	0	**0**
>20%	0	0	0	0	**0**
Total	**26**	**3**	**1**	**0**	**30**

Weighted Kappa test for category agreement: <0.10

Slide A3	*First reading*				
Second reading	0-5%	6-10%	11-20%	>20%	***Total***
0-5%	4	5	2	0	**11**
6-10%	3	2	4	0	**9**
11-20%	3	3	3	1	**10**
>20%	0	0	0	0	**0**
Total	**10**	**10**	**9**	**1**	**30**

Weighted Kappa test for category agreement: 0.16

Slide A4	*First reading*				
Second reading	0-5%	6-10%	11-20%	>20%	***Total***
0-5%	0	0	0	0	**0**
6-10%	0	2	2	1	**5**
11-20%	1	2	5	5	**13**
>20%	0	1	3	8	**12**
Total	**1**	**5**	**10**	**14**	**30**

Weighted Kappa test for category agreement: 0.36

Slide A5	*First reading*				
Second reading	0-5%	6-10%	11-20%	>20%	***Total***
0-5%	3	1	1	0	**5**
6-10%	2	3	3	0	**8**
11-20%	2	2	5	3	**12**
>20%	1	1	2	1	**5**
Total	**8**	**7**	**11**	**4**	**30**

Weighted Kappa test for category agreement: 0.27

11-20% and >20%, and for atypical lymphocytes the categories were 0-10%, 11-30%, 31-60% and >60%. When the paired categorised data were cross-tabulated and weighted *kappa* values for agreement determined a number of important observations became apparent. Only one of the five slides (A1) in the band cell study showed an acceptable degree (*kappa*>0.8) of intra-observer reproducibility (*table 3*).
For the other four slides (A2 to A5), an inability for many individual observers to reproducibly observe and quantify band cell components was clearly apparent. Similar analyses (*table 4*) of the five slides in the atypical lymphocyte study showed that the majority of observers were unable to reproducibly assess this abnormality. Category agreement was very poor and a significant number of paired results showed quantitative revision of two categories or more.
Analysis of category trends (*table 1*) for the two slide series showed that the presence or absence of left shift or IG flags had no influence on observer band cell assessments as the downward versus upward category revisions were evenly balanced (i.e. reflecting observer inconsistency rather than bias). The findings for atypical lymphocytes were, however, somewhat different. The two slides with no flags (B2 and B4) both showed balanced category revisions whereas two (B1 and B3) of the three slides with atypical lymphocyte flags showed clear evidence of upward category revision (i.e. the reporting of higher numbers). The remaining slide (B5) with an atypical lymphocyte flag did not show any apparent category trend (*table 1*). However, closer examination of the results for this particular slide (*table 4*) revealed that six of the seven observers who in the first examination recorded atypical lymphocyte estimates of $\leq$30% revised their estimates upwards when the slides were examined the second time. Virtually all of the downward revisions for this sample were from >60% to 31-60% and thus of lesser significance.

Discussion

There are many literature reports that detail the limitations of morphological analysis and consistency of leukocyte population estimates, particularly when the cell types concerned are defined by criteria that are relatively subjective. However, as far as we are aware this is the first study which has specifically evaluated the potential influence of observer bias (subconscious or otherwise) introduced by awareness of instrument flagging information. The design of this study, whereby the Second Examination of the band cell and atypical lymphocyte slide sets was accompanied by an analyser report with leukocyte differential and flagging information, was designed to provide

table 4 Intra-observer agreement for first and variant lymphocyte examination.

Slide B1	*First reading*				
Second reading	0-10%	11-30%	31-60%	>60%	***Total***
0-10%	6	0	0	0	**6**
11-30%	5	3	5	0	**13**
31-60%	4	2	1	0	**7**
>60%	2	1	1	0	**4**
Total	**17**	**6**	**7**	**0**	**30**

Weighted Kappa test for category agreement: 0.19

Slide B2	*First reading*				
Second reading	0-10%	11-30%	31-60%	>60%	***Total***
0-10%	3	3	0	2	**8**
11-30%	2	3	2	1	**8**
31-60%	1	3	3	1	**8**
>60%	3	1	0	2	**6**
Total	**9**	**10**	**5**	**6**	**30**

Weighted Kappa test for category agreement: 0.12

Slide B3	*First reading*				
Second reading	0-10%	11-30%	31-60%	>60%	***Total***
0-10%	10	2	1	0	**13**
11-30%	5	3	2	0	**10**
31-60%	1	2	2	0	**5**
>60%	1	1	0	0	**2**
Total	**17**	**8**	**5**	**0**	**30**

Weighted Kappa test for category agreement: 0.34

Slide B4	*First reading*				
Second reading	0-10%	11-30%	31-60%	>60%	***Total***
0-10%	8	3	2	0	**13**
11-30%	3	1	3	1	**8**
31-60%	1	1	1	0	**3**
>60%	0	2	2	2	**6**
Total	**12**	**7**	**8**	**3**	**30**

Weighted Kappa test for category agreement: 0.33

Slide B5	*First reading*				
Second reading	0-10%	11-30%	31-60%	>60%	***Total***
0-10%	0	0	0	1	**1**
11-30%	0	1	1	1	**3**
31-60%	3	1	2	6	**12**
>60%	1	1	3	9	**14**
Total	**4**	**3**	**6**	**17**	**30**

Weighted Kappa test for category agreement: 0.07

some insights into whether or not such information could subconsciously bias morphologist's individual assessments. If the appearance of a specific flag led to a biased increase in the recorded number of band

cells or atypical lymphocytes, then this would be reflected by an upward trend in categorised results. Conversely, the absence of the respective flags would lead to a downward trend if morphologists were biased.
Imprecision of conventional microscope differentials results from the combined effects of inherent (Poisson) statistical limitations and observer inconsistencies. Observer subjectivity is particularly relevant to the delineation of abnormal populations such as band cells and variant lymphocytes where there remains considerable variability despite attempts to provide workable morphological definitions. Recommendations for standardising morphological criteria for band cells have largely failed[4, 15, 16], and the situation with variant lymphocytes is even less satisfactory because the diversity of morphological changes in reactive and malignant conditions fundamentally prevents the use of empirical morphological guidelines. Thus, while it is true that observers will agree and consistently recognise obvious morphological disturbances such as cytoplasmic basophilia, nuclear eccentricity or nucleolation, there is much less agreement when these changes are more subtle (e.g., cytoplasmic or nuclear pleomorphism).
Band cells and variant lymphocytes are cell types that are often viewed as indicators of possible bacterial or viral infection when their frequency or number in blood samples exceeds the generally accepted upper normal limits of 5% or 10% respectively. It is apparent however that this theoretical clinical potential would be equally compromised by either a failure in recording the presence of atypical cells or, alternatively, for a morphological reviewer to suggest the presence of atypical cells when in fact they do not exist.
This study evaluated inter- and intra-observer variability of band cell and variant lymphocyte differential estimates by using a somewhat novel experimental approach. The initial part of the study essentially confirmed the perceptions of most morphologists that there is poor concordance between individual observers when designating these leukocyte populations. As mentioned earlier, these inconsistencies are to some extent a consequence of the Poisson error associated with a 100 cell differential. However, our finding that the inter-observer agreement level for the minor population of immature granulocytes (metamyelocytes and myelocytes) in slide A4 was very good strongly suggests that it is specifically the morphological subjectivity of less well-defined leukocyte fractions that is the main contributor to variability.
It can of course be reasonably argued that when the morphological definition(s) of any specified leukocyte population is inadequate or difficult to apply, then the resulting morphological subjectivity is likely to be high. Moreover, when subjectivity is high it is quite conceivable that non-morphological factors could have a significant influence on observer

decisions with respect to leukocyte differential categorisation. This essentially refers to the potential impact of conscious or subconconscious observer bias on final outcomes that may result from (for example) prior awareness of clinical diagnosis, patient symptomology or abnormal flagging alerts provided by haematology analysers. Consequently, it is necessary to consider these aspects when interpreting our findings of poor intra-observer reproducibility for individual morphologists who reviewed the same slides on two separate occasions. If this intra-observer variation was due to Poisson statistics and random morphological inconsistencies, then one would expect to find no significant trend change between the first and second morphological appraisals. If on the other hand, a distinct trend change was observed then this would strongly suggest that the analyser report and flagging information accompanying the second morphological assessment influenced or biased the observer.

Our paired analysis of band cell estimates suggested that supplementary flagging information had little or no influence on observer bias, nor did it positively affect the consistency of reported results. In contrast, it was clear that when analyser flags indicated the presence of atypical or variant lymphocytes that a significant degree of observer bias could be introduced.

In conclusion, these results suggest a morphological dilemma. If morphological reviews of band cells and variant lymphocytes are undertaken with no prior awareness of haematological or clinical information, then the final report and interpretation may reflect the ‘idiosyncrasies’ of the observer as well as statistical limitations. On the other hand, if the morphologist has access to an analyser report and flagging information, then it will have no effect on the ‘randomness’ of the band cell determination but it might induce observer bias in the variant lymphocyte estimate. Either way, the resulting reported information could be clinically misleading and thus counter-productive. On the basis of our findings, we do not recommend the routine determination and reporting of band cell and variant lymphocyte differentials unless unequivocally present in increased numbers. Moreover, the limited accuracy and clear inconsistency of quantifying these leukocyte fractions suggest that a simple appended comment of ‘normal’ or ‘increased’ may be preferable and less ambiguous.

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References

1 Houwen B. The differential cell count. Lab Hematol 2001;7:89-100
2 Rümke CL. Imprecision of ratio-derived differential leukocyte counts. Blood Cells 1985;11:311-314
3 Bain BJ. Blood cells: A practical guide. 3d ed. Blackwell Sciences, Oxford, 2002
4 Cornbleet PJ, Novak R. Lack of reproducibility of band neutrophil identification despite the use of uniform identification criteria. Lab Hematol 1995;1:89-96
5 Miale J. Laboratory Medicine: Hematology 6th ed. St Louis, CV Mosby 1982
6 Glassy E. Color Atlas of Hematology: Illustrated Field Guide Based on Proficiency Testing. Northfield, IL, College of American Pathologists, 1998
7 Lee GR, Foerster J, Lukens J et al. Wintrobe's Clinical Hematology. 10ed. Baltimore, Williams & Wilkins. 1999
8 Cornbleet PJ. Clinical Utility of the band cell. Clin Lab Med 2002;22:101-136.
9 Cox CJ, Habermann TM, Payne BA, Klee GG, Pierre RV. Evaluation of the Coulter Counter Model S-Plus IV. Am J Pathol 1985;84:297-306
10 Warner BA, Reardon DM, Marshall DP. Automated haematology analysers: a four way comparison. Med Lab Sci 1990;47:285-296
11 Krause JR. Automated differentials in the hematology laboratory. Am J Pathol 1990;4:S11-S16
12 Buttarello M, Lorenz C, Gadotti M, Tottalori E, Valentini A, Rizotti P. Evaluation of four automated hematology analyzers. Am J Pathol 1992;3:245-352
13 van der Meer W, Dinnissen JWB, de Keijzer MH, Scott CS. Comparative evaluation of Abbott Cell-Dyn CD3700, Bayer ADVIA 120 and Sysmex XE-2100 automated leucocyte differentials and flagging. Lab Haematol 2002;8:102
14 Pierre RV. Leukocyte differential counting. In 'Practical Laboratory Hematology' (ed JA Koepke). Churchill Livingstone,1991
15 Bacus JW. The observer error in peripheral blood cell classification. Am J Clin Pathol 1973;59:223-228
16 Novak RW. The beleagured band count. Clin Lab Med 1993;13:895-901

Chapter 4

Does the band cell survive the 21th century?

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Eur J Haematol 2006;67:251-254

Abstract

Objectives

The differentiation of white blood cells is a worldwide-accepted method to obtain medical information. The conventional microscopic differential however, is a laborious and expensive test with a low statistical value. Especially for band cell identification there is a wide range of variance. In this report we describe the intervariability of band cell enumeration.

Methods

From a septic patient, an EDTA anticoagulated blood sample was obtained and a smear was made and stained (May-Grünwald Giemsa). A PowerPoint presentation was made twice of 100 random cells and sent to 157 different hospital laboratories in The Netherlands for a leukocyte differential. In the first survey, neutrophils were differentiated in segmented and band neutrophils whereas in the second survey no discrimination was made between segmented and band neutrophils.

Results

The first survey was responded by 68% of the laboratories (756 individuals) and the second survey by 73% of the laboratories (637 individuals). The laboratory mean values of the segmented neutrophils were 42.9% (sd: 7.8, range 22-64%) and 69.9% (sd: 1.4, range 62-72%) for the first and second survey, respectively. For the individual technicians the values of the segmented neutrophils were 43.9% (sd: 11.2, range 15-72%) and 70.0% (sd: 2.0, range 59-77%) for the first and second survey, respectively.

Conclusions

Because of the enormous variation of band cell counting we recommend to cease quantitative reporting of band cells, especially since the results only have a clinical relevance in a limited number of pathological circumstances.

Introduction

The differentiation of white blood cells is a worldwide-accepted method to obtain information about diagnosis, follow-up and treatment of illness. However, the microscopic differentiation is labour intensive and inaccurate[1]. Enumeration of band cells has low clinical utility, but clinicians still use the number of band cells as a parameter for infection[2]. Although the descriptive definition of a band cell is well documented[3-5], the ability of individual technicians to consistently apply these descriptive guidelines is actually quite poor[2]. Lack of consistent interpretation of the band cell identification criteria leads to considerable variation in reference ranges[6-8].

Modern haematology analysers are able to generate automated five-part differentials (expressed in relative as well as absolute counts) and resolve many of the statistical limitations of the microscopic differential[9, 10]. Technical improvements have lead to expansion of the morphological subtypes of cell classes, e.g. immature granulocytes,[11, 12] that can be recognised. Moreover, the general reliability of automated leukocyte differentials has significantly reduced conventional microscopic reviews. Flagging for morphological abnormalities, however, still has little sensitivity[13]. In addition, flagging for abnormalities may lead to overestimation of the number of abnormalities. Remarkably, this is not the case for band cells, probably due to the enormous inter- and intravariation in band cell enumeration[14]. Recently, an automated image recognition system has been developed and evaluated. This may reduce microscopic examination of blood smears[15]. However, traditional microscopy will continue to be used in routine haematology in combination with automated image recognition systems. Further advances in the technology of automated image recognition systems can be expected to create new challenges for the blood cell image interpretation[16].
External quality assurance programs for microscopic leukocyte differential counts is needed to improve morphological performance and uniform assessment of blood cells. Fixed, unstained blood smears are sent to participating laboratories and differentiated by technologists. All results are evaluated and reported to the participants. In this report we describe the intervariability of band cell enumeration. To exclude sample variation we have made two PowerPoint presentations containing both 100 different leukocytes, which were sent to 157 hospital laboratories. For the first survey, neutrophils had to be differentiated in segmented and band neutrophils whereas in the second survey no discrimination was asked between segmented and band neutrophils.
The aim of this study is to prove that the intervariability of enumeration of band cells is controversial.

Materials and methods

A blood smear was prepared from a septic patient and stained according to the method of May-Grünwald Giemsa. From the slide 100 leukocytes were randomly selected and micro photographed and each cell was presented in a PowerPoint presentation. This presentation was sent to 157 hospital laboratories in the Netherlands for assessment of each leukocyte type. All data were processed in Excel files for further analysis.
After one year another set of 100 images was derived from the same blood smear and processed into a PowerPoint presentation. This

presentation was sent to the same laboratories requesting to differentiate the leukocytes. However, participants were now asked to count band cells as neutrophils. If band cells were present, it was requested to report this by means of a plus, independent of the amount of band cells.
For each cell the accordance was investigated as a percentage of similar assessment for each cell.
For data analysis Anova unpaired t-test with Welch's correction was used.

table 1. The mean values of leukocyte types reported through the laboratories in the first and second survey

Leukocyte type	First survey (n=106)				Second survey (n=114)			
	Mean (%)	Sd (%)	Range (%)	CV (%)	Mean (%)	Sd (%)	Range (%)	CV (%)
Monocytes	12.4	2.2	3-15	17	8.4	2.9	0-13	34
Lymphocytes	6.6	1.5	3-15	23	15.8	1.2	8-19	7
Segmented	42.9	7.8	22-64	18	69.9	1.4	62-72	2
Band cells	31.4	7.6	11-52	24	-	-	-	-
Metamyelocytes	4.4	1.6	0-10	36	2.9	1.4	0-9	48
Myelocytes	0.8	0.9	0-0	100	1.8	2.1	0-12	100
Eosinophils	0.0	0.1	0-0	-	0.0	0.1	0-0	-
Basophils	1.0	0.1	0-2	10	0.0	0.0	0-0	-
otherwise	0.5	0.6	0-3	100	1.2	1.8	0-11	100

Results

Of 157 laboratories, 106 (68%) responded to the first survey and 114 (73%) to the second test. For the first survey 756 individual results were received and 637 from the second survey. 104 Laboratories responded to both the first and the second survey.

Laboratory means.

The laboratory mean of segmented neutrophils count for the first survey was 42.9% with a standard deviation (sd.) of 7.8%, a range of 22 - 64% and a coefficient of variation (CV) of 18%. The laboratory mean neutrophil count (=segmented neutrophils and band cells) for the second survey was 69.9% with a sd. of 1.4%, a range of 62 - 72% and a CV of 2%. See table 1 for the results of the other cell categories. Results from both surveys were compared for every category using the unpaired t-test with Welch's correction for each cell type. This resulted in a statistical significant difference (p<0.0001) for neutrophils.

Individual means.

The individual technician mean segmented neutrophil count, for the first survey was 43.9% with a sd. of 11.2%, a range of 15 - 72% and a CV of 25%. The individual technician mean neutrophil count for the second survey was 70.0% with a sd. of 2.0%, a range of 59 - 77% and a CV of

3%. See table 2 for the results of the other cell categories. Results from both surveys were compared for every category and resulted in a significant difference (p<0.0001) for neutrophils.

Individual observer data for each cell.

First survey. As each observer was shown the same set of cells, the agreement between participants in this study in categorizing each cell could be analysed. For the segmented neutrophils >75% accordance between participants was achieved for 27 cells. For band cells 14 cells showed a better than 75% accordance. For 19 segmented neutrophils and 13 band cells accordance was less than 75% (*figure 1*).

Second survey. In the second survey, participants showed more than 75% accordance for 69 out of 70 neutrophils. Only one neutrophil cell reached less than 75% accordance in categorizing. The presence of band cells was reported in 632 out of 637 observations (99.2%).

Additionally, monocytes and (meta)myelocytes showed standard deviations of 0.9-2.9% (*table 1*) for the laboratory values and 1.4 - 3.9% (*table 2*) for the individual values.

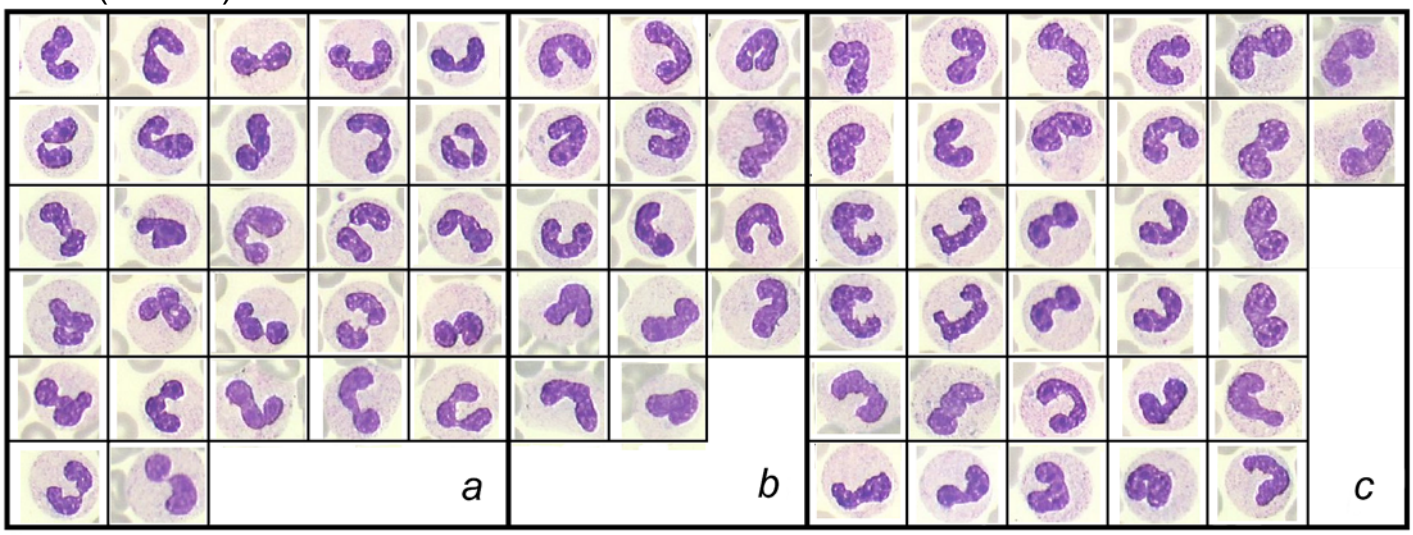

fig 1

Overview of the neutrophils from the first survey. 1a: 27 Cells with an accordance of >75% for segmented neutrophils. 1b: 14 Cells with an accordance of >75% for band neutrophils. 1c: 32 Cells with an accordance <75% for segmented neutrophils or band neutrophils.

Discussion

The leukocyte differential count is an important component of the routine full blood count [17]. Detection of a granulocytic left shift in microscopic differential counts is often used as an indicator for infection or sepsis.

table 2 The mean values of leukocyte types reported through by individuals in the first and second survey

Leukocyte type	First survey (n=756)				Second survey (n=637)			
	Mean (%)	Sd (%)	Range (%)	CV (%)	Mean (%)	Sd (%)	Range (%)	CV (%)
Monocytes	12.4	3.1	2-19	25	8.4	3.9	0-21	46
Lymphocytes	6.6	1.8	1-18	27	15.8	1.9	7-23	12
Segmented	43.9	11.2	15-72	25	70.0	2.0	59-77	3
Band cells	30.6	11.0	4-64	36	-	-	-	-
Metamyelocytes	4.2	2.4	0-15	57	3.0	2.6	0-15	87
Myelocytes	0.8	1.4	0-13	100	1.6	2.5	0-16	100
Eosinophils	0.0	0.1	0-1	-	0.0	0.2	0-3	-
Basophils	1.0	0.2	0-3	20	0.0	0.1	0-1	-
otherwise	0.5	1.1	0-8	100	1.2	2.3	0-15	100

Traditionally, a left shift has been defined as an elevated neutrophil band count[2, 18]. In the leukocyte index band cells are used to calculate the immature to total neutrophil ratio. According to some authors the leukocyte index could provide supplement information in sepsis diagnosis[19]. However, other authors dispute this statement[20]. Despite explicit national and international guidelines, a uniform discrimination between band cells and segmented neutrophils has never been achieved[21]. In our study we found a wide variation in results, both between individuals and laboratories. In previous statistical studies Rümke developed a model for differentials[1] showing that a 100-cell differentiation is by definition a statistically unreliable sample check. His model is based on sample variance: observation of the same cells by different observers is mere coincidence. In our investigation all technicians observed identical cells and this should therefore reduce sample variance.

Differentiation of neutrophils in segmented and band cells lead to a standard deviation of 11.2% for segmented neutrophils and 11.0% for band cells in a range of 15-72% and 4-64%, respectively. These results differ significantly from those of the other cells, where statistically differences between the results of the mean from different observers were mainly due to the low number of the cells in the PowerPoint presentation.

This study shows that qualitative reporting of band cells leads to a considerable reduction in standard deviation to circa 2% for neutrophils in the range of 59-77%. Of all 637 observers, 632 (99,2%) reported the presence of band cells.

The clinical utility of band cells in children older than 3 months of age is poor [2]. Nevertheless, enumeration of band cells is still required for the Rochester criteria and for the calculation of the immature to total

neutrophil ratio. But due to the enormous interobserver variation of band cell enumeration, one should reconsider the use of quantifying the number of band cells. Results of band cell numbers should at least be interpreted with great care. The number of band cells may suggest a left shift, which may be a sign for infection[21]. Other neutrophil precursors such as metamyelocytes are less controversial and can provide the same information. However, the accordance for monocytes and (meta)myelocytes was poor. This may be due to the inter variation of the staining method. The technicians may be attuned to another colour intensity for monocytes and (meta)myelocytes. For this reason especially these cells were confused with each other. With the introduction of other diagnostic parameters for the detection of sepsis or infection (e.g. CRP, procalcitonin, cytokines), the need to differentiate band cells from segmented neutrophils will diminish. Anticipating on these new developments, we recommend ceasing enumeration of band cells in daily clinical practice.

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References

1 Rümke CL. Imprecision of ratio-derived differential leukocyte counts. Blood Cells 1985;11:311-314
2 Cornbleet PJ. Clinical Utility of the band cell. Clin Lab Med 2002;22:101-136
3 Miale J. Laboratory Medicine: Hematology 6th ed. St Louis, CV Mosby 1982
4 Glassy E. Color Atlas of Hematology: Illustrated Field Guide Based on Proficiency Testing. Northfield, IL, College of American Pathologists 1998
5 Lee GR, Foerster J, Lukens J et al. Wintrobe's Clinical Hematology. 10th ed. Baltimore, Williams & Wilkins 1999
6 Harrison TR, Fauci AS. Harrison's principles of internal medicine, 14th ed. New York, Mc Graw-Hill health division 1998
7 Dale DC, Federman DD. Scientific American medicine. New York, Scientific American 1997
8 Novak RW. The beleagered band count. Clin Lab Med 1993;13:895-993
9 Krause JR. Automated differentials in the hematology laboratory. Am J Pathol 1990;4:S11-S16
10 Buttarello M, Gadotti M, Lorenz C, Tottalori E, Valentini A, Rizotti P. Evaluation of four automated hematology analyzers. Am J Pathol 1992;3:345-352
11 Ansari-Lari MA, Kickler TS, Borowitz MJ. Immature granulocyte measurement using the Sysmex XE-2100 : relationship to infection and sepsis. Am J Clin Pathol 2003;120:795-799

12 Nigro KG, O'Riordan MA, Molloy EJ, Alsh MC, Sandhaus LM. Performance of an automated immature granulocyte count as a predictor of neonatal sepsis. Am J Clin Pathol 2005;123:618-624
13 van der Meer W, Dinnissen JWB, de Keijzer MH, Scott CS. Comparative evaluation of Abbott Cell-Dyn CD3700, Bayer ADVIA 120 and Sysmex XE-2100 automated leucocyte differentials and flagging. Lab Haematol 2002; 8: 102
14 Van der Meer W, de Keijzer MH, Scott CS. Automated flagging influencing the inconsistency and bias of band cell and atypical lymphocyte morphological differentials. Clin Chem Lab Med 2004;4:371-377
15. Swolin B, Simonsson P, Backman S, Löfqvist I, Bredin I, Johnsson M. Differential counting of blood leukocytes using automated microscopy and a decision support system based on neural networks – evaluation of DiffMaster™ Octavia. Clin Lab Haem 2003;25:139-147
16 Tatsumi N, Pierre RV. Automated image processing: past, present, and future of blood cell morphology identification. Clin Lab Med 2002;22:299-315
17 Houwen B. The differential cell count. Lab Hematol 2001;7:89-100
18 Manroe BL, Rosenfeld CR, Weinberg AG, Browne R. The neonatal blood count in health and disease. I. Reference values for neutrophilic cells. J Pediatr 1979;95:89-98
19 Rosenfeld CR. Band neutrophil counts in neonates. J Pediatr 1995;3:504-506
20 Schelonka RL, Yoder BA, desJardins SE, Hall RB, Butler J. Clinical and laboratory observations: differentiation of segmented and band neutrophils during the early newborn period. J Pediatr 1995;127:298-300
21 Seebach JD, Morant R, Rüegg R, Seifert B, Fehr J. The diagnostic value of the neutrophil left shift in predicting inflammatory and infectious disease. Am J Clin Pathol 1997;107:582-591

Chapter 5

The variant assessment of variant lymphocytes

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J Clin Pathol *in press*

Abstract

It is commonly accepted that classification of abnormal lymphocytes in peripheral blood smears contributes to a rapid diagnosis of lymphocyte disorders. In this paper we describe the inconsistent morphological assessment of subtypes of lymphocytes in a nationwide morphology query. A PowerPoint presentation was made of 100 at random micro photographed white blood cells. This presentation was sent to 157 laboratories in The Netherlands resulting in a response of 73% (114 laboratories; 671 results of individual technicians). Participants were asked to subdivide the lymphocytes in normal, atypical lymphocytes, plasma cells, prolymphocytes or (lympho)blasts. There were 56 pictures of lymphocytes. Of these lymphocytes only seven cells were unanimously classified as normal lymphocytes, for 30 cells two different subtypes were mentioned, for 11 cells three different subtypes were mentioned, for 7 cells four different subtypes were made and in one case even all five subtypes were mentioned. One cell was showed twice in the PowerPoint presentation and this resulted in an inconsistent classification for 210 out of 671 observers (31%). Support of additional clinical information may improve the classification of abnormal lymphocytes.

Introduction

An examination of a blood smear may be requested by physicians or initiated by laboratory staff as diagnostic aid when a lymphoproliferative disorders or mononucleosis is suspected[1]. The classification of lymphocyte disorders is complex because of the various manifestations of these disorders[2,3]. Three major groups can be distinguished: reactive lymphocytosis, premalignant neoplastic disorders of lymphocytes and neoplastic disorders of lymphocytes[4]. Lymphocyte disorders may be accompanied by abnormal lymphocyte morphology. Therefore, it is important to recognize and report abnormal lymphocytes, such as atypical lymphocytes and lymphoblasts. The ability of individual technicians, however, to recognize abnormal lymphocytes consistently is quite poor[5]. There are no standardised definitions regarding the morphology of the various cells, and interpretation is based on individual experience and dependent on the availability of additional clinical information. Often, transitional forms between lymphocytes and plasma cells are seen in the blood of patients with viral infections. These cells are variously known as atypical lymphocytes, lymphocytoid plasma cells or plasmacytoid lymphocytes[6]. The so-called atypical lymphocyte is a non-neoplastic lymphocyte seen in the peripheral blood, and appears to be a non-specific response to stress from a variety of disorders[7]. Small

lymphocytes become larger in size and become capable of dividing. These atypical lymphocytes vary in morphology and surface markers[8,9]. Hoagland developed criteria for diagnosis of infectious mononucleosis comprising ≥50% of the white blood cell differential with atypical lymphocytes accounting for ≥10% of the total WBC count[10]. The morphology of lymphoblasts is even more complicated, as morphological appearance varies from small cells with scanty cytoplasm and coarse chromatin to large cells with an abundance of vacuolated cytoplasm and fine chromatin[11].
The morphological assessment of the peripheral blood smear remains a valued diagnostic tool [1], despite the considerable interobserver variation in interpretation. New techniques such as automated recognition systems may reduce the microscopic review of blood films in the near future[12]. Nevertheless, the relevance of the morphological evaluation of abnormal lymphocytes can be questioned, considering the large variation in reported results.
In this paper we demonstrate the general inconsistency in reporting whether an abnormal lymphocyte is (non-neoplastic) reactive, neoplastic or an arte fact.

Material and Methods

From a blood smear of an orthopaedic patient (female, age 3 years) with a lymphocytosis, which was normal for a child with that age, 100 white blood cells were randomly selected, micro photographed and processed in a PowerPoint presentation. This PowerPoint presentation was sent to 157 different hospital laboratories in The Netherlands and a leukocyte differential was requested. All participants were asked to differentiate the lymphocytes in normal lymphocytes, atypical lymphocytes, plasma cells, prolymphocytes or (lympho)blasts. Participants did not have any information of the patient.
All results were processed in Excel and for each laboratory and individual technician the mean differential was calculated. For each lymphocyte subtype the degree of concordance was calculated.

Results

Of the 157 Laboratories 114 responded (73%) with a total of 671 individual results. The overall mean lymphocyte count (including cell subtypes) was 56%. For 7 cells there was a concordance of >90% for normal lymphocyte (*figure 1a*), which means that more than 90% of the individual observers called these cells normal lymphocytes. For the other 49 lymphocytes there was no concordance in subtyping at all. In

majority, lymphocytes (29 cells) were classified as normal or atypical (*figure 1b*). One cell (*figure 1c*) was classified as a normal lymphocyte or a plasma cell. Nine cells were classified as normal or atypical or prolymphocyte (*figure 1d*) and two cells as normal or atypical or plasma cell (fig. 1e). For seven cells four different subtypes were mentioned (*figure 1f and g*), and one cell was attributed for five different assessments (*figure 1h*).
One cell (*figure 2*) was shown twice in the PowerPoint presentation: in 210 out of 671 the same cell was classified by the same observer as another subtype.

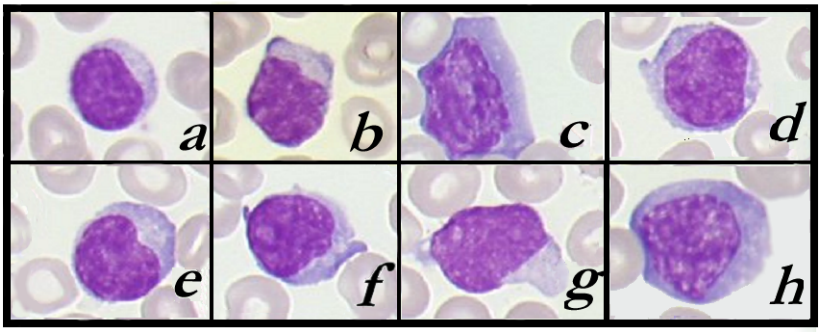

fig 1
Examples of the different lymphocytes. Lymphocytes classified as: a; lymphocyte with >90% accordance, b; normal or atypical, c; normal or plasma cell, d; normal, atypical or prolymphocyte, e; normal, atypical or plasma cell, f; normal, atypical, prolymphocyte or plasma cell, g; normal, atypical, prolymphocyte or blast, h; normal, atypical, prolymphocyte, plasma cell or blast.

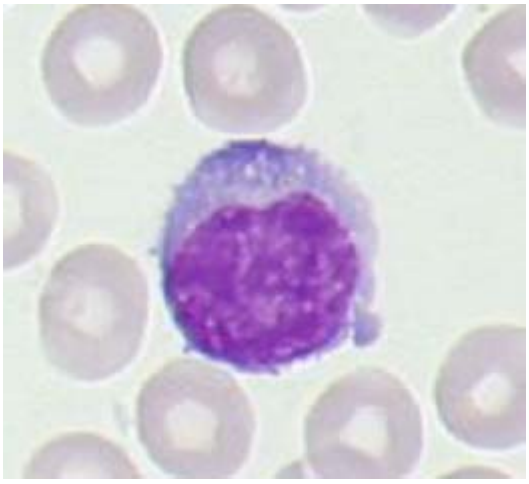

fig 2
The duplicate lymphocyte

Discussion

Even in the age of molecular analysis and sophisticated laboratory tests, the blood smear remains an important diagnostic tool[1]. The recognition of abnormal lymphocytes in blood smears can contribute to a rapid

diagnosis of various diseases, both reactive and (premalignant) neoplastic, and enables rapid therapeutic intervention. Some rare diseases may be diagnosed faster by a critical review of the lymphocytes, such as I-cell disease[13]. The need for recognition of abnormal lymphocytes should not be underestimated. The morphology of lymphocytes is complex: Cell size, amount and colour of cytoplasm, as well as shape and chromatin structure of the nucleus, must be combined to come to a good characterisation of the lymphocyte. Diagnosis of infectious mononucleosis may be based on Hoagland's criteria. However, previous studies have questioned the percentage of patients with serologic evidence using these criteria[14]. Unfortunately, a uniform definition of abnormal lymphocytes is lacking. A confusing terminology is used for abnormal lymphocytes, e.g. variant lymphocytes, atypical lymphocytes and even combinations of several cells are used, e.g. monocytoid lymphocytes and plasmacytoid lymphocytes. In this study we demonstrated the variability in classification of abnormal lymphocytes and would recommend interpreting and reporting abnormal lymphocytes with care. One cell was shown twice in the PowerPoint presentation (*figure 2*), and remarkably 31% of the morphologists was not able to reproduce their previous classification. This illustrates the problem of uniform and consistent reporting of the subtypes of abnormal lymphocytes even more clearly.
In spite of the fact that highly qualified technologists spend large amount of time on manual microscopy, clinicians base their diagnosis and treatment often on other information. This is because clinicians have access to physical findings, X-rays, cultures etc.[15] We recommend providing additional clinical information, which should lead to a better interpretation of blood cell morphology. However, additional information of the automated haematology analysers could lead to overestimation of abnormal lymphocytes[5].

Acknowledgements
We are grateful to the many technicians and institutions that performed the blood cell morphology. We also would like to thank the SKML (Dutch Society of Quality Assurance in Medical Laboratory Diagnostics) for their support.

References

1 Bain BJ. Diagnosis from the blood smear. N Engl J Med 2005;353:498-507
2 Saulsbury FT. B cell proliferation in acute infectious lymphocytosis. Pediatr Infect Dis J 1987;6:1127-1129
3 Hutchinson RE, Kurec AS, Davey FR. Lymphocytic surface markers in lymphoid leukemoid reactions. Clin Lab Med 1988;8:237-245
4 Lichtmann MA in Williams Hematology 5ed. McGraw-Hill Publishing Company 1995
5 Van der Meer W, Scott CS, de Keijzer MH. Automated flagging influencing the inconsistency and bias of band cell and atypical lymphocyte morphological differentials. Clin Chem Lab Med 2004;4:371-377
6 Paraskevas in Wintrobes Clinical Hematology 11ed. Lippinkot Williams & Wilkins 2004
7 Simon MW. The atypical lymphocyte. Int Pediatr 2003;18:20-22
8 Inman DR, Cooper EH. The relation of ultrastructure to DNA synthesis in human leukocytes. Acta Haemat 1965;33:257-278
9 Shiftan TA, Mendelsohn J. The circulating “atypical” lymphocyte. Hum Pathol 1978;9:51-61
10 Hoagland RJ. The clinical manifestations of infectious mononucleosis: a report of two hundred ceases. Am J Med 1960;240:21-29
11 Bain BJ; Blood cells: a practiacal guide. 2ed. Blackwell Sciences, Oxford, 1995
12 Swolin B, Simonsson P, Backman S, Löfqvist I, Bredin I, Johnsson M. Differential counting of blood leukocytes using automated microscopy and a decision support system based on artificial neural networks-evaluation of DiffMaster Octavia. Clin Lab Haem 2003;25:139-147
13 van der Meer W, Jakobs BS, Bocca G, Smeitink JAM, Schuurmans Steckhoven JH, de Keijzer MH. Peripheral blood lymphocyte appearance in a case of I cell disease. J Clin Pathol 2001;54:724-726
14 Brigden ML, Au S, Thompson S, Brigden S, Doyle P, Tsaparas Y. Infectious mononucleosis in a outpatient population. Arch Pathol Lab Med 1999;123:875-881
15 Houwen B. White blood cell morphology in the balance. Lab Hematol 2005;11:79-82

Chapter 6

Peripheral blood lymphocyte appearance in a case of I-cell disease

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J Clin Pathol 2001;54:724-726

Abstract

In general, peripheral blood smears are performed to obtain information with regard to various morphological features as an aid in the diagnosis of infection or malignancy.

Here, we present a patient with I cell disease (Inclusion-cell disease), a fatal lysosomal storage disorder caused by a defect in an enzyme responsible for the transfer of mannose-6-phosphate ligands to precursor lysosomal enzymes. As a consequence, most lysosomal enzymes are transported outside the cell instead of being correctly targeted into the lysosomes, resulting in storage of macromolecules in lysosomes.

I cell disease, with its heterogeneous clinical presentation, can be diagnosed by the presence of intracellular vacuole-like inclusions in lymphocytes and fibroblasts, high serum lysosomal enzyme activities and a defect of N-acetylglucosamine-1-phosphotransferase.

In this report we describe the morphological aspects of peripheral lymphocytes in a blood smear of a patient, the first clue to the final diagnosis of I-cell disease. The observed vacuole-like inclusions in lymphocytes of this patient were negative for Periodic Acid-Schiff (PAS) and Sudan Black B staining, in contrast to earlier reports.

Introduction

I cell disease is a rare autosomal recessive lysosomal storage disorder, first described in 1967[1], which manifests at birth and slowly progresses in a fatal outcome at approximately 4 years of age[1-3]. The disease, although heterogeneous in its clinical presentation, has some characteristic features, namely: psychomotor retardation, skeletal abnormalities, shortness of stature and a Hurler-like appearance. Intracellularly, a generalised storage of macromolecules occurs in lysosomes due to multiple lysosomal enzyme deficiencies. Since lysosomes are the cellular organelles in which macromolecules are degraded, these macromolecules accumulate leading to abnormal inclusions in cells such as lymphocytes. Therefore, diagnosis of I cell disease can be accomplished by detection of cytoplasm inclusion bodies in lymphocytes or cultured skin fibroblasts together with multiple lysosomal enzyme activity abnormalities. Several groups[1, 2, 4, 5] have investigated the ultrastructural features in I cell disease. A common finding in these studies was the various cytoplasm inclusion bodies found in several types of cells. Furthermore, these inclusion bodies contained macromolecules, which could be stained with Sudan Black B (lipids) and periodic acid Schiff (PAS; glycogen)[4].

Here, we describe a peripheral blood smear from a patient with vacuole-like inclusions in the cytoplasm of lymphocytes, which led to an early diagnosis of I cell disease. The diagnosis of I cell was finally confirmed by lysosomal enzyme activity measurements in plasma.

Case report

This girl was born after an uneventful pregnancy at 38 weeks gestation as the first child of nonconsanguineous Turkish parents. The mother had 3 miscarriages before at 3 months of gestational age. The birth weight of the child was 2330 g. She was dysmorphic at birth, but on request of both parents no further investigation was done. At the age of 56 days she was admitted to our hospital with severe respiratory insufficiency, caused by bronchopneumonia. Artificial ventilation was needed for four days. Physical examination revealed the following abnormalities: coarse facies, thick eyelids, hypertelorism, low implanted ears, narrow forehead, micro- and retrognatia, laterally bowed legs and rocker bottom feet. The liver was 3 cm and the spleen was 1 cm palpable. The activity of alkaline phosphates in blood was increased to as much as 3240 U/L (reference value: up to 350 U/L, age and sex related). Most of the alkaline phosphatase originated from the bones. Serum phosphate, serum calcium and total protein were decreased (0.78 mmol/L, 1.10 mmol/L and 47 g/L respectively).

Haemocytometry of the blood provided a value of haemoglobin, 4.4 mmol/L; leukocytes, 7.8 x 10^9/L; and platelets, 172 x 10^9/L.

During her stay at the paediatric ward the patient drank poorly and her increase in body weight was marginal. After three days she was readmitted to the intensive care because of respiratory insufficiency and cardiogenic shock. She was treated with digoxin and diuretics. When she was clinically stable she was discharged from hospital. From then, the parents withdrew her from follow-up.

Methods

Air dried blood smears were stained using standard methods of May-Grünwald Giemsa, Sudan Black B and PAS.

Na-citrate blood (3.8% vol/vol) was centrifuged at 750x*g* for 10 min at room temperature. Plasma was decanted and the cells were fixed in 2% gluteraldehyde phosphate buffer (140.4 mmol/L NaCl, 13.8 mmol/L Na_2HPO_4, 1.9 mmol/L NaH_2PO_4: pH 7.4) at 4°C. After three hours the buffy coat was removed, sliced and post fixed in 1% osmiumtetroxide for one hour at room temperature. After dehydration in graded concentrations of alcohol, the blocks were embedded in Epon. The ultra

thin sections (80 nm) were contrasted with uranyl acetate and lead nitrate and examined with a JEOL 1200 EX/II electron microscope[6].
Lysosomal enzyme activities in plasma and fibroblasts were measured as described previously[7].

Results

The blood smear revealed 88% lymphocytes, 8% granulocytes and 4% monocytes. Approximately 25% of the lymphocytes were atypical and appeared to have grey blue inclusions (size 1-2 μm) with a vague azurophilic granulation in the centre (*figure 1*).

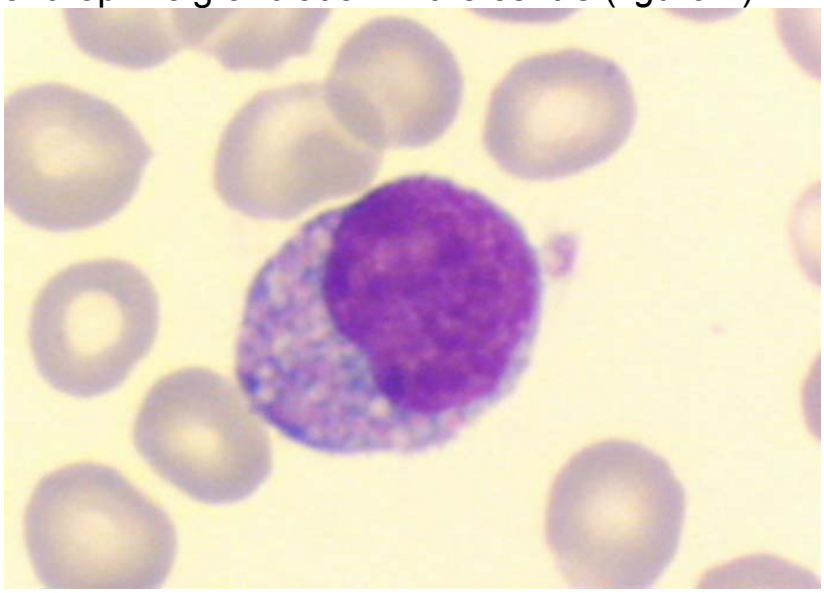

fig 1
A lymphocyte with many vacuole-like inclusions. The cytoplasm is stained blue, the vacuole-like inclusions are stained pink (900x).

In disrupted normal lymphocytes, only a nuclear ghost was visible, however in case of disrupted abnormal lymphocytes (a consequence of preparing a blood smear), the vacuole-like inclusions were still visible (*figure 2*). These vacuole-like inclusions might suggest a viral infection. Staining with Sudan Black B to identify a lipid storage disease and PAS to detect a glycogen storage disease gave negative results.
Electron microscopy of the blood cells revealed a large number (up to 20) of cytoplasmic vacuoles, some of which had no visible content, although most had an aggregation of small globular or possible tubular structures. In addition, a round osmiophilic structure was found in most cells (*figure 3*).
The diagnosis was finally confirmed by the low activities of β-D-galactosidase (8 nmol/h/mg protein; ref.value: 600-1,500), N-acetyl-β-D-glucosaminidase (1,715 nmol/h/mg protein; ref.value: 8,000-38,000) and

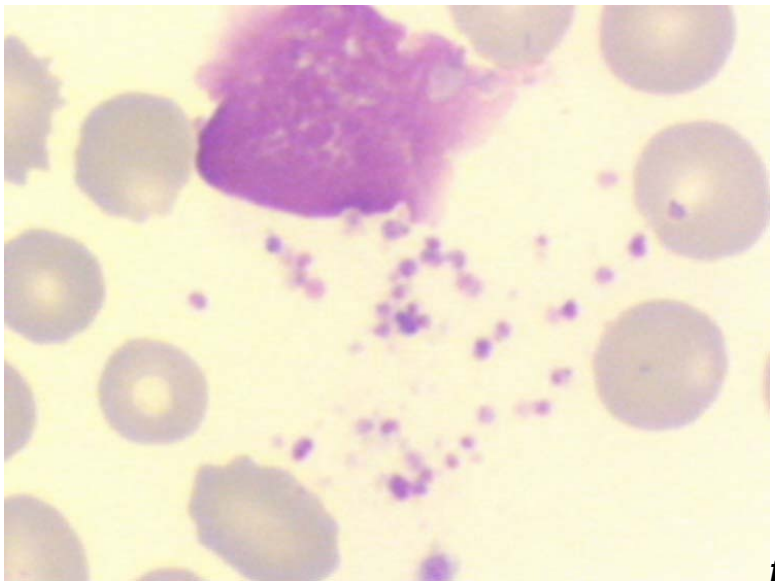

fig 2
A disrupted cell with around the nuclear remnant, the released inclusions (900x).

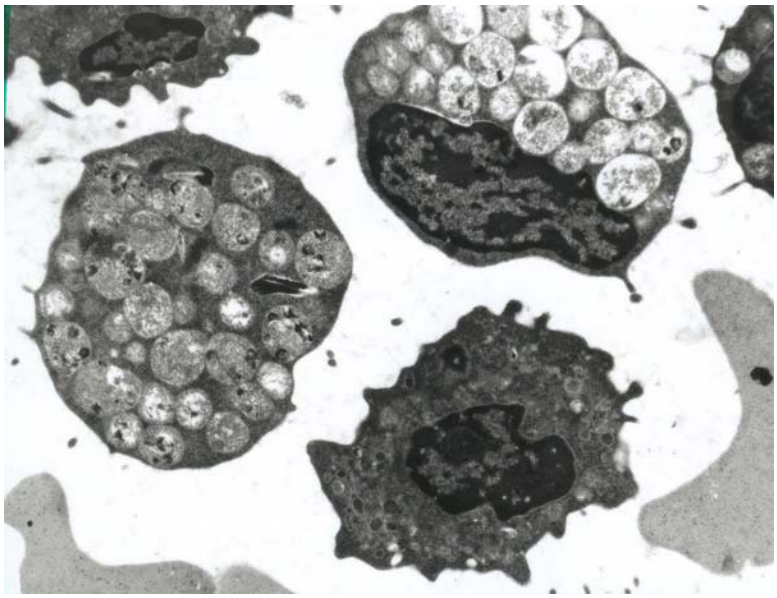

fig 3
Electron microscopic presentation of vacuoles in lymphocytes with the round osmiophilic structures (15,000x)

α-D-mannosidase (16 nmol/h/mg protein; ref.value: 200-720) in fibroblasts and the high activities of β-D-galactosidase (117 nmol/h/mg protein; ref.value: <20), N-acetyl-β-D-glucosaminidase (26,582 nmol/h/mg protein; ref.value: 900-7,000) and α-D-mannosidase (7,000 nmol/h/mg protein; ref.value: 50-350) in plasma. These findings are characteristic for an I-cell disease.

Discussion

I-cell disease is evident from birth showing a gradual degeneration. The disease has an autosomal recessive mode of inheritance.

Although several authors have described vacuoles or inclusions in the lymphocytes of patients with I-cell disease[3, 8, 9], images of both electron microscopy and light microscopy were only occasionally presented[10]. In our case, the vacuoles appeared to be inclusions. Neutrophils and monocytes had a normal appearance in our patient's blood smears. Electron microscopy of the lymphocytes revealed that the inclusion bodies had a single membrane and most of them contained an osmiophilic structure. However, in contrast to Leroy et al[8], PAS and Sudan Black B staining were negative. After disruption of the cells these inclusions were clearly visible, whereas in the case of normal lymphocytes only a nuclear ghost was visible.

Although many authors described the I-cell disease, only Koga and co-workers[3] and Leroy and co-workers[8] have mentioned vacuoles or inclusion bodies in blood cells. This report confirms these findings. However, in contrast to Koga[3] we did not find vacuoles in neutrophils[3, 8]. Careful microscopic observation of peripheral blood smears may lead to the discovery of rare abnormalities in blood such as intracellular metabolite accumulation. The presence of abnormal granulation or vacuolisation of lymphocytes can facilitate a rapid diagnosis of some inherited metabolic diseases.

Acknowledgement

We thank Mr Frank Rietveld for technical assistance with the electron microscopy and the co-workers of the laboratory for neurology for measurement of the lysosomal enzymes.

References

1 Okada S, Owada M, Sakiyama T, Yutaka T, Ogawa M. I-cell disease: clinical studies of 21 Japanese cases. Clin Gen 1985;28:207-215

2 Kamiya M, Tada T, Kuhara H et al. I-cell disease A case report and review of the literature. Acta Pathol Jpn 1986;36:1679-1692

3 Koga M, Ishihara T, Hoshii Y, Uchino F, Matsuo K, Yamashita Y. Histochemical and ultrastructural studies of inclusion bodies found in tissues from three siblings with I-cell disease. Pathol Int 1994;44:223-229

4 Leroy JG, De Mars RI. Mutant enzymatic and cytological phenotypes in cultured human fibroblasts. Science 1967;157:804-806

5 Güngör N, Coskun T, Akçören Z, Çaglar M. I-cell disease, a case report and review of the literature. Turk J Ped 1994;36:145-152

6 Schuurmans Steckhoven JH, van Haelst UJGM, Joosten EMG, Gabreels FJM. Ultrastructural study of so-called curvilinear bodies and fingerprint structures in lymphocytes in late-infantile amaurotic idiocy. Acta Neuropath 1976;35:295-306
7 De Jong JGN, Wevers RA, van den Berg CJM, Liebrand-van Sambeek MLF, van Rens AAET, Roelofs HGM. Diagnostiek van lysosomale stapelingsziekten. Nederlands Tijdschrift Klinische Chemie 2000;25:13-27
8 Leroy JG, Spranger JW, Feingold M, Opitz JM, CrockerAC. I-cell disease: A clinical picture. J Pediat 1971;79:360-365
9 Kitagawa H, Toki J, Morimoto T et al. An autopsy case of I-cell disease. Am J Clin Pathol 1991;96:262-266
10 Bain BJ. Blood cells: a practiacal guide. 2^{d} ed. Blackwell Sciences, Oxford, 1995

Chapter 7

Pseudoplatelets: a retrospective study of their incidence and interference with platelet counting

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J Clin Path 2003;56:772-774

Abstract

Spurious platelet counts can be found in acute leukaemia's, caused by fragmentation of blood cells. Microscopic examination of a blood smear should be performed to detect the presence of these so-called pseudoplatelets. In case of their presence, the platelet count should be corrected in time because of the important clinical consequences of this finding in these patients.

K_3EDTA anticoagulated blood was measured on an automated blood cell counter, and a blood smear was made and stained according the May-Grünwald Giemsa method for microscopic observation. A 500 cell/particle differentiation was performed and the automated platelet count was corrected.

We studied the incidence of pseudoplatelets in 169 patients with acute leukaemia. Pseudoplatelets were detected in 43 cases (25.4%), and 7 cases (4.1%) were re-classified as having a major bleeding risk (platelet count $<15x10^9$/L).

We emphasize the importance of the morphologic observation of platelets in patients with acute leukaemia and the development of a routine screening method for the detection of pseudoplatelets.

Introduction

Haemorrhagic diathesis is a common manifestation of acute leukaemia and is usually caused by thrombocytopenia[1]. Haemostatic disturbances observed in patients with acute leukaemia can be related to changes in vascular function, failure of the haematopoietic system, liver dysfunction, increased fibrinolysis or disseminated intravascular coagulation (DIC)[2]. In such patients, a spurious platelet count due to red cell fragmentation[3] or fragmentation of white blood cells[4-11] has been reported previously. Stass e.a.[5] reported spurious elevation of automated platelet counts in a patient with hairy cell leukaemia, with at the microscopic counting a significantly lower number of platelets. Armitage e.a.[6] described a patient with acute leukaemia with artificially elevated platelet counts due to circulating fragments of leukaemic cells. Malcolm e.a.[7] reported a case of spurious thrombocytosis in a patient with acute myelocytic leukaemia, with at microscopic observation of the blood smear a thrombopenia: The automated platelet count in this patient was ten times higher as compared to the microscopic count. Stass e.a.[8] reported a case of spurious platelet counts due to cell fragmentation in a patient with a poorly differentiated lymphoma: After chemotherapy the number of platelet-like fragments increased. Hammerstrom[9] reported a patient with a newly diagnosed acute myeloid leukaemia (AML: FAB M5) with DIC for which the patient was treated with low dose heparin. Although the

platelet count was 129x10^9/L, the patient developed an intracerebral bleeding and died. Staining the blood smears with platelet-specific antigen resulted in only 4% positive cell particles, whereas one third of the particles showed identical staining characteristics as the leukaemia cells, indicating their leukaemic origin. Sugimoto e.a. [10] reported a case of AML (FAB M2) with cell fragments resembling giant platelets positive for myeloperoxidase. Finally, Li e.a.[11] reported a case with secondary acute monocytic leukaemia with tumour lysis syndrome: Numerous fragments of leukaemic cells resulted in a falsely elevated platelet count. Moreover, apoptotic cells with pycnotic nuclei were seen, suggesting a relationship between apoptosis and pseudoplatelets.
In the present study we investigated 169 patients with leukaemia, both *de novo* diagnosed and relapsed (*table 1*). Furthermore, we looked at the bleeding tendency of patients with pseudoplatelets who are classified into the risk group for bleeding disorders after correction of the automated platelet count.

table 1. Distribution of leukaemia type, age, onset/relapse and sex in the group of 169 patients

	mean age (min-max)	Onset/relapse	Male/female
Total	35 (2-83)	131/38	93/76
ALL	15 (2-64)	53/7	40/20
AML-M0	48 (32-79)	1/3	1/3
AML-M1	53 (9-79)	11/4	9/6
AML-M2	50 (9-82)	17/1	10/8
AML-M3	40 (19-59)	10/0	3/7
AML-M4	51 (14-83)	17/3	13/7
AML-M5	44 (7-70)	7/3	3/7
AML-M6	53	1/0	1/0
AML-M7	26 (2-51)	2/0	0/2
Not specified	45 (11-75)	12/16	13/15

To exclude that pseudoplatelets are an intrinsic result of slide preparation method, both the correlation of pseudoplatelets with smudge cells and pseudoplatelets with leukocytosis was studied.

Materials and methods

Blood was obtained by venapuncture or by finger prick and anticoagulated with K_3EDTA. Within four hours, the blood was measured for complete blood cell count (CBC) and a blood smear was made by the wedge method. The slides were stained according the May-Grünwald Giemsa method. Stained slides were embedded and stored in a dark place at room temperature.

CBC was performed by an automated analyser: H*3 from Bayer (Tarrytown, USA) or Sysmex NE-8000 from Toa (Kobe, Japan). The principle of platelet counting is based on flow cytometry (H*3) or impedance (NE-8000). Both platelets and erythrocytes are counted in the same channel: discrimination between platelets and erythrocytes is made on particle size.
The patients included in the study were diagnosed with acute leukaemia at onset or at relapse with blast cells in the peripheral blood. The various types of leukaemia, onset/relapse and age and sex of the patients are listed in table 1. The acute leukaemia's were further specified according the FAB-classification[12] with the aid of immunofenotyping and cytochemistry. Of each sample a 500 particle differentiation was made (platelets, pseudoplatelets, leukocytes and smear cells) and the percentage of pseudoplatelets was calculated. In case of high WBC, the platelets and pseudoplatelets were counted separately. Arbitrarily, if more than 5% pseudoplatelets were present the smear was considered positive for the presence of pseudoplatelets. These positive samples were also reviewed by an independent expert. Both manual countings were averaged out and used to correct the platelet count. As cut off value for serious bleeding risk we have used $15x10^9$/L: The mean of $10x10^9$/L and $20x10^9$/L, as published previously[13, 14].

Results

In the blood smears of 43 (25%) out of 169 patients pseudoplatelets were found: 32 (30%) smears out of 107 patients with AML and in 11 (18%) smears of 62 patients with ALL (*table 2*). The morphology of pseudoplatelets was comparable with agranular platelets, but with a deeply stained cytoplasm in accordance with the cytoplasm of malignant cells (figure 1). Note that in these patients red cell fragments were not found. Of this group of 43 patients, a total number of 11 patients was retrospectively considered as positive for major bleeding events (*table 3*). The platelet count obtained with a haematology analyser varied from $10x10^9$/L to $75x10^9$/L in these patients, while the corrected platelet count ranges from $2x10^9$/L to $15x10^9$/L, with a mean difference of $20x10^9$/L or 40%. Two patients with a corrected platelet count of $10x10^9$/L and $3x10^9$/L were not included because of technically poor blood smears.
There was no correlation between pseudoplatelets and smudge cells (R^2: 0.14) nor for the pseudoplatelets and leukocytosis (R^2: 0.04) (*figure 2*).

table 2. Incidence of the presence of pseudoplatelets in relation with the different types of leukaemia

Type	*Subtype*	*Total number of patients*	*Number of patients with pseudoplatelets*	*Percent*
ALL		**62**	**11**	**18**
AML		**107**	**32**	**30**
	AML-M0	4	0	0
	AML-M1	15	5	33
	AML-M2	18	4	22
	AML-M3	10	2	20
	AML-M4	20	3	15
	AML-M5	10	3	30
	AML-M6	1	0	0
	AML-M7	2	0	0
	Not further specified	28	1	4
Total		**169**	**43**	**25**

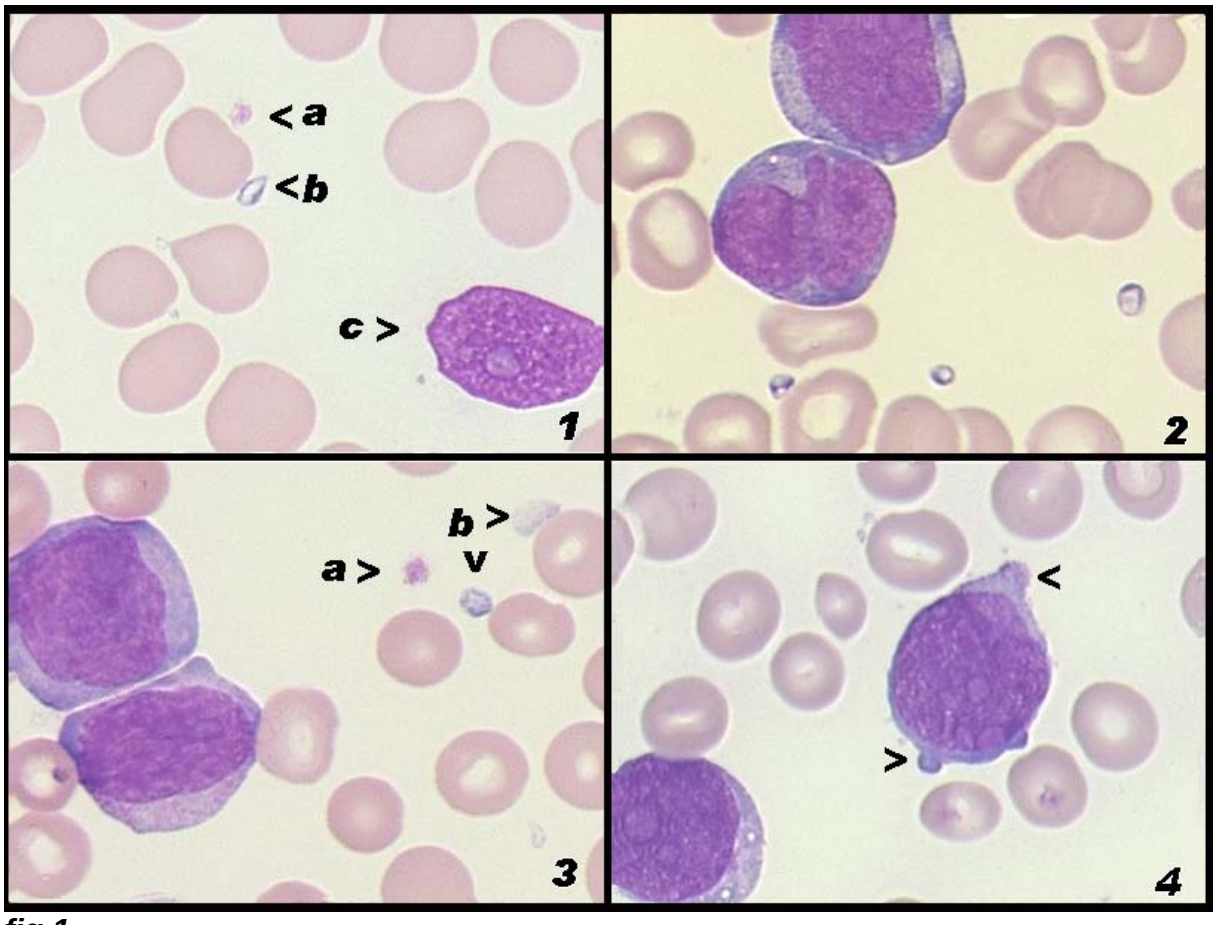

fig 1
*Morphologic aspects of pseudoplatelets: **1** AML-M1 with true platelet (**a**) pseudoplatelet (**b**) and smear cell (**c**), **2** AML-M5 with three pseudoplatelets and one blast cell, **3** AML-M2 with true platelet (**a**), two pseudoplatelets (**b**) and two (pro)blast cells, **4** ALL with pseudoplatelet budding (arrowhead) of a lymphoblast.*

Discussion

Nowadays, haematology analysers are able to produce platelet counts with great precision and accuracy. Even in the very low range (below 15 x 10^9/ L), the counts are usually reliable. However, in certain cases these analysers produce erroneous platelet results. Pseudotrombopenia is a well-known phenomenon and via various procedures (e.g. recounting platelets in citrated blood) the laboratory is able to produce a correct result. We report an opposite phenomenon with important clinical implications: pseudothrombocytosis or at least obvious overestimation of the real number of platelets in patients with acute leukaemia. Due to their shape and size, haematology analysers add a number of undefined particles to the platelet cluster. In some cases, this may even lead to the masking of a (possible life-threatening) thrombopenia and consequently the withholding of proper medication or other crucial supportive measures.

table 3 Overview of the patients with a corrected platelet count of 15 x 10^9/L or less. In parenthesis the range is given.

No	Type	Age/sex	Automated platelet count x 10^9/L	Corrected platelet count x 10^9/L (range)
1	ALL	43/m	10	2 (1-2)
2	AML-M1	44/m	24	12 (10-13)
3	AML-M1	55/m	57	14 (12-16)
4	AML-M1	9/m	10	6 (5-6)
5	AML-M1	63/m	28	14 (10-18)
6	AML-M2	74/v	75	15 (10-20)
7	AML-M2	46/m	13	8 (7-9)
8	AML-M2	81/v	48	8 (5-10)
9	AML-M3	22/v	23	14 (12-16)
10	AML-M5	56/v	14	8 (7-9)
11	AML-M5	24/m	21	9 (9)

The undefined particles (or pseudoplatelets) are not formed due to mechanical destruction during the preparation of the smear. No correlation was found between the number and presence of smudge cells and the number and presence of pseudoplatelets, indicating a different origin.

To establish a proper platelet count, we suggest that the blood smears at presentation and at unexpected bleeding disorders in patients with acute leukaemia (*de novo* or relapsed) should be examined for pseudoplatelets. In the presence of pseudoplatelets, the automated platelet count should be corrected. In the present study, in more than 25% of the described patients the platelet counts had to be corrected. As

in almost 5% of the patients this resulted in a re-classification of the bleeding risk, the finding of pseudoplatelets has important consequences for the clinical management of patients with acute leukaemia.

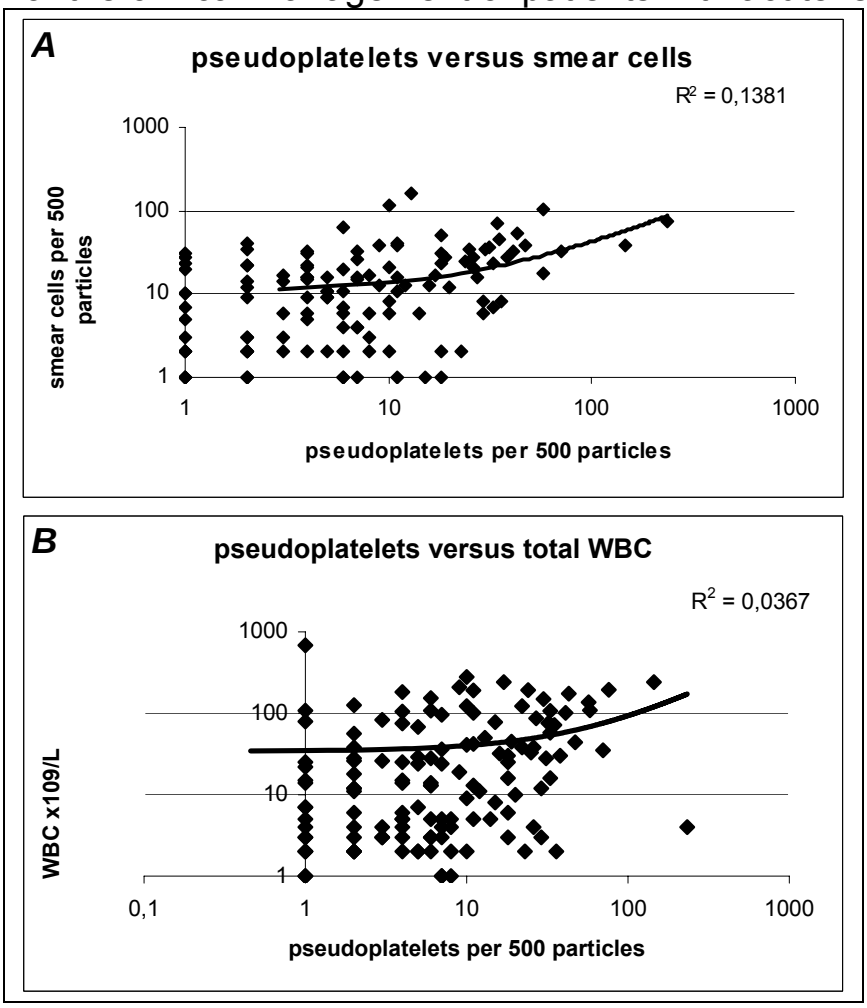

fig 2
Correlation of pseudoplatelets with smear cells and WBC

References

1 Wilde JT, Davies JM. Haemostatic problems in acute leukaemia. Blood Rev 1990;4:245-251
2 Törnebohm E, Blombäck M, Lockner D et al. Bleeding complications and coagulopathy in acute leukaemia. Leuk Res 1992;16:1041-1048
3 Savage RA, Lucas FV, Hoffman GC. Spurious trombocytosis caused by red blood cell fragmentation. Am J Clin Pathol 1983;1:144
4 Hanker JS, Giamarra BL. Neutrophil pseudoplatelets: Their discrimination by myeloperoxidase demonstration. Science 1983;220:415-417
5 Stass SA, Holloway ML, Slease RB et al. Spurious platelet counts in hairy cell leukemia. Am J Clin Pathol 1977;68:530-531
6 Armitage JO, Goeken JA, Feagler JR. Spurious elevation of the platelet count in acute leukemia. J Am Med Assoc 1978; 239:433-434
7 Malcolm ID, Monks P, Katz M. Spurious thrombocytosis in acute myelocytic leukemia. N Engl J Med 1978;298:1260-1261

8 Stass SA, Holloway ML, Peterson V et al. Cytoplasmatic fragments causing spurious platelet counts in the leukemic phase of poorly differentiated lymphocytic lymphoma. Am J Clin Pathol 1979;71:125
9 Hammerstrom J. Spurious platelet counts in acute leukaemia with DIC due to cell fragmentation. Clin Lab Haemat 1992;14:239-243
10 Sugimoto T, Saigo K, Ryo R. Giant platelet-like cell fragments produced from abnormal promyelocytes in acute myelogenous leukemia. Rinsho Byori 1998;46:182-185
11 Li S, Salhany KE. Spurious elevation of automated platelet counts in secondary acute monocytic leukemia associated with tumor lysis syndrome. Arch Pathol Lab Med 1999;11:1111-1114
12 Bennett JM, Catowsky D, Daniel MT et al. Proposal for the classification of acute leukaemia's. Br J Haematol 1976;33:451-458
13 Beutler E. Platelet transfusions: the 20,000/μL trigger. Blood 1993;81:1411-1413
14 Wandt H, Frank M, Ehninger G et al. Safety and cost effectiveness of a $10x10^9$/L trigger: A prospective comparative trial in 105 patients with acute myeloid leukemia. Blood 1998;91:3601-3606

Chapter 8

Simultaneous determination of membrane cd64 and hla-dr expression by blood neutrophils and monocytes using the monoclonal antibody fluorescence capability of a routine haematology analyser

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Abstract

This study reports the design of an immunofluorescent method for the co-determination of neutrophil CD64 (PMN-CD64), monocyte CD64 (MON-CD64) and monocyte HLA-DR (MON-Ia) expression with the Cell-Dyn CD4000 haematology analyser. Normal PMN-CD64, MON-CD64 and MON-Ia expression, defined as the mean ±2SD of 25 healthy adults after correction for isotype control staining, corresponded to 17-67, 515-1045 and 170-670 AFU, respectively. Analytical reproducibility determined by duplicate analysis of 12 random samples revealed good assay consistency for all three analysed antigens, with day to day variation in normal subjects being relatively minor in significance. CD4000 PMN-CD64 values showed a good correlation with flow cytometry although short term (12 hours) stability studies suggested an *in vitro* trend for increasing PMN-CD64 antigen expression with progressive storage. Observed ranges of PMN-CD64, MON-CD64 and MON-Ia for 109 randomly-selected clinical samples were 31-1058, 307-2843 and 10-876 AFU. Abnormal PMN-CD64 and MON-CD64 shared the same trend (upregulation) while abnormal monocyte MON-Ia was characterised by declining expression. Normal PMN-CD64 was only seen with normal (45/52) or intermediate (7/52) MON-CD64, while high PMN-CD64 was mostly associated with intermediate (18/22) or high (3/22) MON-CD64. MON-Ia expression was largely independent (p=0.04) of PMN-CD64 although marked decreases in MON-Ia were invariably associated with intermediate or high PMN-CD64. MON-Ia expression was inversely related (p<0.0001) to absolute granulocyte counts, and patients with high PMN-CD64 were more likely (8/25) to have in excess of 10% band cells compared to samples with normal/intermediate PMN-CD64 (0/84). When compared to C-Reactive Protein (CRP), high PMN-CD64 and MON-CD64 were always associated with an increased CRP concentration, but minor proportions of samples with normal PMN-CD64 (11/52) or normal MON-CD64 (11/65) could also have an increased CRP. The procedures described in this communication overcome a number of limitations associated with flow cytometry, and co-determination of CD64 and HLA-DR antigen expression may provide additional insights into patient heterogeneity in the assessment of suspected sepsis compared to CD64 analysis alone.

Introduction

There are many reports regarding the potential of measuring neutrophil membrane CD64 (PMN-CD64) for the diagnostic assessment of sepsis[1-6]. The membrane molecule defined by monoclonal antibody CD64 is a high-affinity receptor (FcγRI) found on normal monocytes and is only

expressed at low levels by normal neutrophils. Upregulation and increased expression of PMN-CD64 appears to be a sensitive marker for early-onset clinical infection in newborn children[6-7], and in adults its use has been variably suggested for differentiating systemic infection from active inflammatory disease[8], monitoring γ-interferon therapy[2] and as an indicator for initiating or discontinuing antibiotic treatment[6]. PMN-CD64 analysis may be particularly useful for assessing sepsis in young children and elderly patients, where haematological parameters such as leukocyte/granulocyte counts, or the presence of immature granulocytes and band cells are relatively uninformative[9-11] and for investigating patients with primary disturbances in neutrophil numbers associated with haematological malignancies or myelosuppressive therapies. All studies to date of PMN-CD64 expression have been performed by flow cytometry using fluorochome-monoclonal antibody conjugates. However, a more efficient approach would be to develop a procedure whereby EDTA-anticoagulated blood samples submitted for FBC analysis could be directly processed for PMN-CD64 as a supplementary procedure by the haematology laboratory. The first aim this study was therefore to examine whether or not a method for the simultaneous measurement of PMN-CD64 and monocyte CD64 (MON-CD64) could be potentially implemented on a routine haematology analyser (Cell-Dyn CD4000; Abbott Diagnostics, Santa Clara, CA, United States). As part of the method design, a second marker (HLA-DR) was included because (in contrast to CD64) its expression by monocytes is reportedly decreased in patients with sepsis[12-14]. Supplementary aims of this study were therefore to determine relationships between relative PMN-CD64 expression and the levels of MON-CD64 and MON-Ia in a randomly-selected series of patient samples where clinical requests for C-Reactive Protein (CRP) had been made, and to examine pattern heterogeneity of CD64/HLA-DR antigen expression in comparison with CRP concentration and absolute granulocyte count.

Materials and methods

CD4000 Analysis of Neutrophil and Monocyte Membrane CD64/HLA-DR: In addition to the standard full blood count (FBC) configuration, the CD4000 optical bench has the capability of measuring FL1 and FL2 fluorescence. These are integrated into a specific assay processing routine for the enumeration of $CD3^+CD4^+$ and $CD3^+CD8^+$ populations [15]. The method used for the co-determination of PMN-CD4, MON-CD64 and MON-Ia expression was based on an adaptation of the CD4000 automated CD3/4/8 assay. In essence, the two reaction vials (CD3/CD4 and CD3/CD8) normally used for the CD3/4/8 counts of a single sample

were substituted with non-anticoagulated Vacutainer tubes containing 100µL whole blood plus 20µL anti-CD64/FITC (IgG1 subclass; Becton Dickinson) and 15µL anti-HLA-DR/PE (IgG2a subclass; Becton Dickinson). The two tubes were processed using the CD4/CD8 assay mode after preliminary incubation at room temperature for 10 min. Antibody-blood ratios were obtained following preliminary titration studies to confirm saturating antibody concentrations, and in this assay configuration the two tubes corresponded to identical CD64/HLA-DR analyses for two different patient samples. On completion of instrument blood sampling and data acquisition (List Mode enabled), raw data files were downloaded to a PC and batch-converted to standard FCS2.0 format with Cell-Dyn Clinical Data Standard (CDS) converter software (CDS Office Suite; Abbott Diagnostics Cell-Dyn R&D Department, Europe) prior to population analysis. This 'in-house' software was designed to generate single-dilution FCS files, readable by standard FCS software/freeware, because Cell-Dyn systems produce multiple files (for each part of instrument analysis) that are stored within a single List Mode file. Converted List Mode files from the CD4000, typically comprising 12k to 20k individual events, allow generation of multiple plot types with parameter options being 7^{o} Intermediate Angle Scatter, 0^{o} Axial Light Loss (size), FL1, FL2 and FL3.

Samples Studied:

To determine control ranges for neutrophil and monocyte antigen expression for the CD4000 method, 25 samples were collected from normal healthy adult volunteers. Supplementary studies were also undertaken to define methodological limitations. Assay reproducibility was determined by duplicate analysis of 12 randomly selected samples, and stability of PMN-CD64 antigen expression during sample storage was assessed by analysing 12 EDTA-anticoagulated samples at 1, 6 and 12 hours after collection. In addition, day to day variation in individual normal subjects was determined by examining neutrophil and monocyte antigen expression with samples from 9 healthy volunteers collected three days apart, and the CD4000 method for PMN-CD64 antigen quantitation was compared with a flow cytometry procedure by analysis of 12 further samples. The flow cytometry method (Beckmann Coulter Epics XL) involved incubation of EDTA anticoagulated blood samples with monoclonal antibodies for 15 minutes, erythrocyte lysis with 0.15M ammonium chloride for 10 minutes, washing and cell resuspension. Using 90^{o} side-scatter and CD45-ECD/CD14-PE/Cy5 fluorescence (Trillium Diagnostics LLC, Maine USA) granulocytes were gated and monocytes excluded. By means of parallel analysis with CD64-FITC and

CD163-PE (Trillium Diagnostics), granulocytes were defined as $CD64^+$ when fluorescent staining exceeded the first log decade.

To further understand the relationships between PMN-CD64, MON-CD64 and MON-Ia expression, a series of 109 EDTA-anticoagulated patient samples were analysed within 6 hours of venesection (after storage at ambient temperature). These were randomly selected from the routine haematology workload and comprised patients for whom turbidimetric measurements of C-reactive Protein (CRP) had been clinically requested on the same day. Full blood counts were obtained with the CD4000 analyser, and stained blood films were reviewed to determine the morphological differential (including percentages of band cells and Immature Granulocytes). In addition, leukocyte integrity was assessed using the automated CD4000 propidium iodide staining procedure[16] and all samples showed >98% viability.

Statistical Analysis:
Relationships between individual measured parameters were variously analysed by logarithmic plots, Passing & Bablok[17] agreement and the Chi-square test (Analyse-It Software Ltd, PO Box 103, Leeds, England).

RESULTS

Methodological Procedures:
Gating of neutrophil and monocyte populations (*figure 1*) with WINMDI flow cytometry software (http://facs.scripps.edu/software.html) was facilitated by primary separation of all collected leukocyte events into $HLA\text{-}DR^+$ and $HLA\text{-}DR^-$ fractions (FL1 versus FL2 plot). Region setting for the neutrophil population was then based on the morphological discrimination of $HLA\text{-}DR^-$ events in the CD4000 optical (0^o versus 7^o) plot. Monocytes were defined as the $HLA\text{-}DR^+$ population (irrespective of the actual level of expression) within the upper area of the CD4000 MAPSS 0^o versus 7^o plot. After region setting, all events associated with each defined leukocyte fraction were analysed in FL1 and FL2 histograms (4-decade log) to obtain the median and geometric means of CD64 and HLA-DR staining intensities (*figures 2 and 3*). As these were numerically similar for all tested samples, the median value, expressed as arbitrary fluorescent units (AFU) was subsequently used for all comparative measurements. Isotype controls were used to determine 'background' fluorescence for each cell population and this was deducted from the median values of sample fluorescence prior to comparative analysis. In addition, FL3 staining (propidium iodide) automatically included in the analytical process by the CD4000[16] was

assessed to confirm that all studied samples had high (>98%) leukocyte viability and no evidence of sample deterioration.

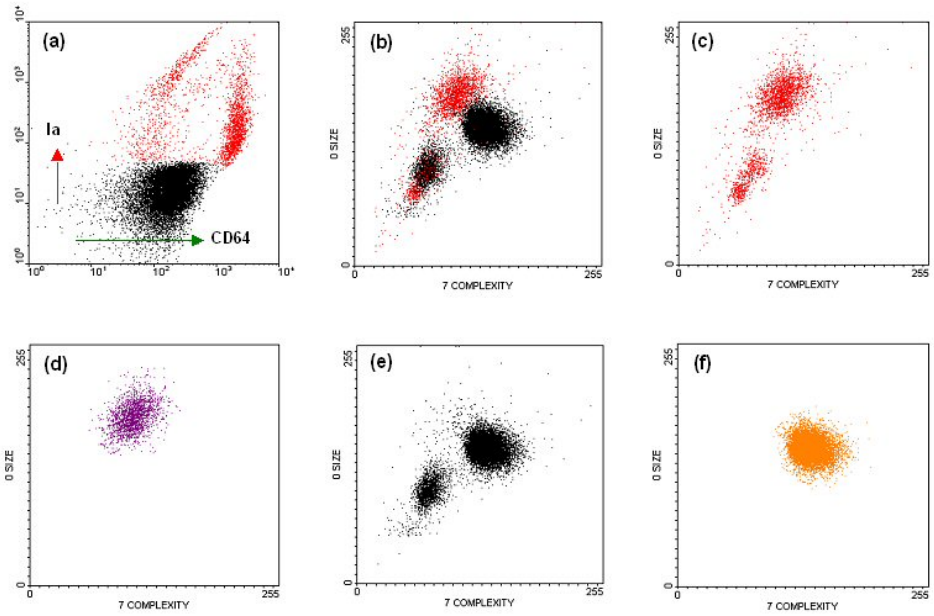

fig 1

Gating procedure for discrimination of neutrophil and monocyte populations. All leukocyte events were examined in an CD64/FL1 versus HLA-DR/FL2 plot (a) and a region set for the HLA-DR$^+$ fraction. The events were then redisplayed in the CD4000 0^o (Size) versus 7^o (Complexity) plot (b) and the HLA-DR$^-$ events excluded (c). A second region (purple) was set around the larger sized monocyte fraction and the other HLA-DR$^+$ events (predominantly B-cells) excluded (d). The resulting gated monocyte fraction was then used for the semi-quantitative determination of CD64 (MON-CD64) and HLA-DR (MON-Ia) expression. The neutrophil fraction was defined by excluding HLA-DR$^+$ events in plot (b) to leave two residual populations (e). A further region gate (orange) was applied to the cluster with the higher Size and Complexity profile and the other HLA-DR$^-$ events (predominantly T-cells) excluded. The resulting neutrophil fraction was then used for the determination of CD64 (PMN-CD64) expression.

Preliminary assessment of FL1 and FL2 data for files with and without fluorescent channel compensation revealed that crossover of FL1 fluorescence (CD64) into the FL2 channel (HLA-DR) had no significant effect on semi-quantitative levels of HLA-DR staining. Therefore in order to simplify the procedure, non-compensated files were used for statistical determinations of median antigen staining.

Control Neutrophil and Monocyte Antigen Expression

Histogram profiles of PMN-CD64 staining intensity showed a normal distribution, and analysis of the 25 normal samples revealed a mean PMN-CD64 of 98 AFU (observed range 83-126). Parallel analyses with an IgG1 isotype control reagent indicated background levels of 56 AFU and this value was subsequently deducted from all PMN-CD64 determinations. Based on the corrected control values (observed minus

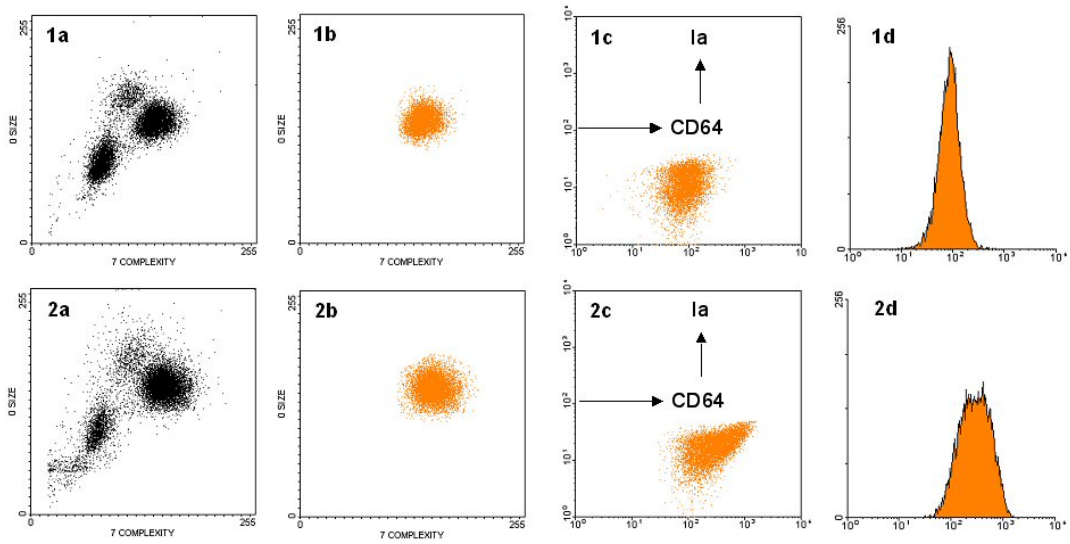

fig 2

Examples of gating procedures and subsequent determination of PMN-CD64 expression. The upper series of samples (1a to d) are for a normal sample with PMN-CD64 staining of 102 AFU while the lower series (2a to 2d) are for a sample with markedly increased PMN-CD64 of 278 AFU. The neutrophil population gating procedure is as detailed in Figure 1 and Results, and the distribution of CD64 staining intensities are shown in histogram form (1d and 2d). While the neutrophil morphological characteristics of the two samples are similar (1b and 2b), the increased expression of CD64 in sample 2 is clearly seen as an extended profile on the CD64 (FL1) axis (2c and 2d). Note the minor tendency for cross-channel fluorescence from FL1 (CD64 axis) into FL2 (Ia axis) at higher levels of PMN-CD64 expression in plot 2c.

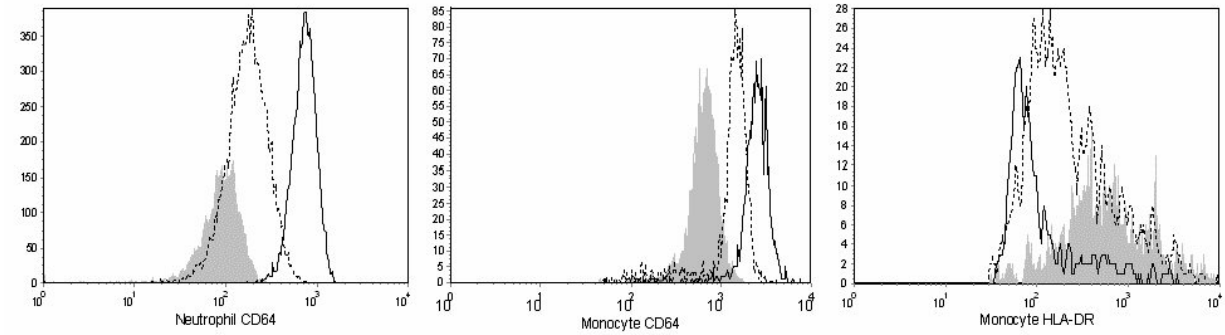

fig 3

Neutrophil CD64 (PMN-CD64), monocyte CD64 (MON-CD64) and monocyte HLA-DR (MON-Ia) histogram staining profiles. The three examples shown in histogram (a) show (uncorrected for isotype controls) median PMN-CD64 staining levels of 90 AFU (solid), 166 AFU (dotted) and 722 AFU (continuous) and correspond to normal, intermediate and high PMN-CD64 staining categories respectively. Histogram (b) shows samples with normal (solid, 673 AFU), intermediate (dotted, 1433 AFU) and high (solid, 2371 AFU) monocyte CD64 (MON-CD64) expression. Histogram (c) are examples with normal (solid, 542 AFU) and intermediate decrease (dotted, 184 AFU) and markedly decreased (continuous, 81 AFU) monocyte HLA-DR (MON-Ia) expression. Note the normal distributions of staining for PMN-CD64 and MON-CD64 compared to the non-normal staining pattern for MON-Ia, and the existence of MON-Ia subpopulation heterogeneity.

background), the normal range for PMN-CD64 defined as the mean ± 2SD (Standard Deviation) corresponded to 17 to 67 AFU. A high level of PMN-CD64 expression (>135 AFU) was therefore defined as a PMN-CD64 value exceeding twice the upper normal limit, while an intermediate increase represented PMN-CD64 expression between 67 and 135 AFU. Using a similar approach for deriving normal MON-CD64, normal, intermediate and high MON-CD64 expression were respectively defined as 515 to 1045, 1046 to 2090 and >2090 AFU after correction for background (isotype control) fluorescence.

In contrast to neutrophil and monocyte CD64, where abnormal trends were associated with antigen upregulation, abnormal monocyte HLA-DR (MON-Ia) was characterised by declining expression. Thus, the isotype-corrected normal range (mean ±2SD) for 25 normal samples corresponded to 170 to 670 AFU, while a marked decrease was defined as less than 50% of the lower normal limit (<85 AFU) and an intermediate decrease as MON-Ia values between 85 and 170 AFU.

Methodological Variables:
Analytical reproducibility was determined by duplicate analysis of 12 random patient samples. Of these, all were evaluable for PMN-CD64 expression and all but one were evaluable for MON-CD64 and MON-Ia expression. The single sample that could not be analysed had very high PMN-CD64 expression (580 AFU) with severe monocytopenia and too few monocyte events for reliable analysis. The results of these duplicate studies (*figure 4*) revealed a high level of assay consistency for all three analysed antigens.
Stability of PMN-CD64 antigen expression during sample storage was assessed by analysing 12 EDTA-anticoagulated samples at 1, 6 and 12 hours after collection. Although there was some individual sample variability, an apparent trend was nevertheless observed. At 1 hour, the mean PMN-CD64 expression was 63.7 AFU and this was normalised to represent a 100% baseline level. At 6 hours, the mean change was +5% (range -7% to +26%) and at 12 hours the mean change was +16% (range 0% to +47%). Further examination of the data revealed that there was no significant decrease in neutrophil viability during the 12 hour period.

Day to day variation in normal individuals was determined by examining neutrophil and monocyte antigen expression with samples from 9 healthy volunteers collected three days apart. The results (*figure 5*) indicated that for PMN-CD64 expression, 8/9 showed similar assay values within the normal control range. Only one subject showed a significant difference

with a PMN-CD64 of 139 AFU (intermediate increase) on day 1 compared to 67 AFU (normal) on day 3. Similar day to day consistency was also seen with MON-CD64 and MON-Ia, with only one individual in each group showing higher expression on day 1 compared to day 3.
Correlation between the CD4000 method and the flow cytometric procedure was assessed by parallel analysis of 12 randomly selected samples. Although the methods differed in the way the data was

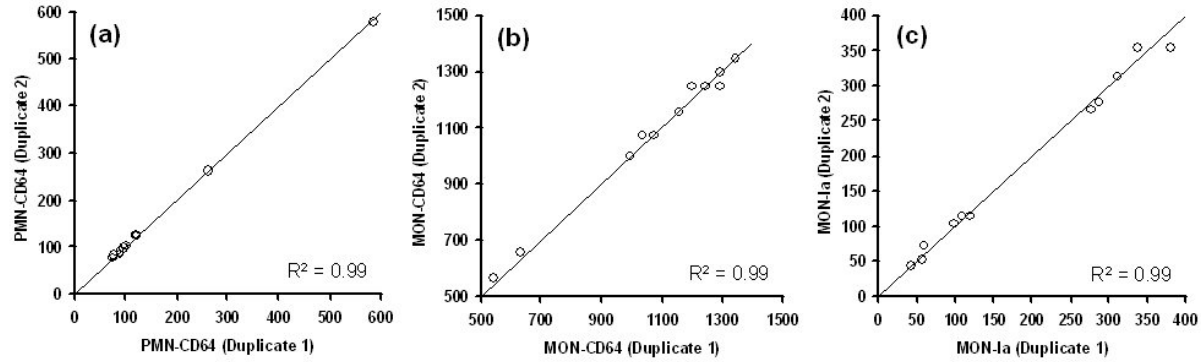

fig 4
Duplicate analysis of 12 randomly selected samples for PMN-CD64, MON-CD64 and MON-Ia expression. For each tested sample, two separate tubes were stained and processed, with the levels of antigen expression (AFU) being determined as detailed in the text. The paired measurements for MON-CD64 and MON-Ia expression exclude one sample with severe monocytopenia where there were too few events for reliable analysis.

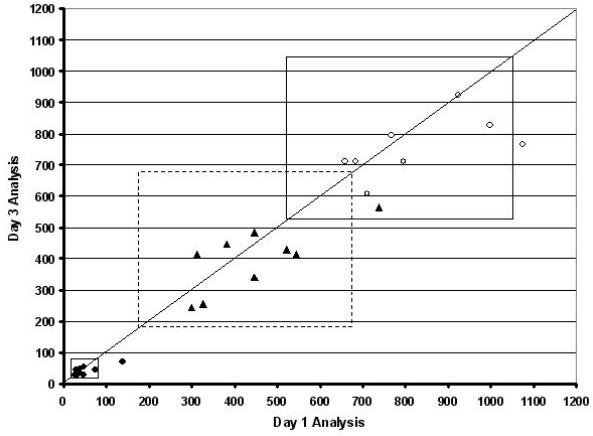

fig 5
Day to day consistency of PMN-CD64 (solid circles), MON-CD64 (open circles) and MON-Ia (solid triangles) expression. Nine healthy subjects were venesected on two separate occasions three days apart and assayed for all three antigens with the CD4000 method. Normal ranges are shown as boxed rectangles, with the data indicating generally good stability of antigen expression. Outliers, corresponding to one of the nine subjects in each of the assay groups, are seen to slightly exceed normal limits on day 1 only of the comparative analyses.

reported, a point which is considered in more detail in the Discussion, there was nevertheless a good correlation with higher CD4000 AFU values being associated with higher percentages of $CD64^+$ neutrophils (*figure 6*).

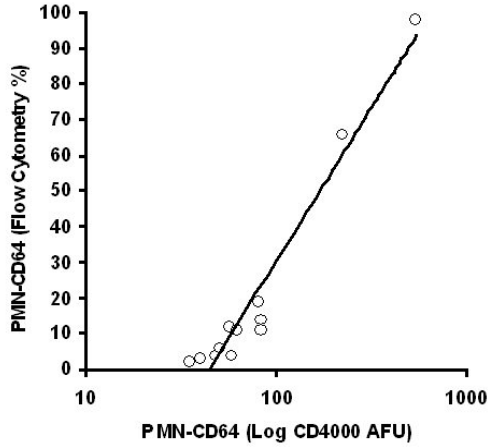

fig 6
Comparison of the CD4000 method for PMN-CD64 expression (Log_{10} AFU) and flow cytometry (percentage $CD64^+$ neutrophils as defined in Materials and Methods) for 12 analysed samples. Although the methods of reporting for the two procedures were different, a good level of correlation (logarithmic trendline) is clearly seen.

Patterns of Neutrophil and Monocyte CD64 and HLA-DR Antigen Expression:
Observed ranges of PMN-CD64, MON-CD64 and MON-Ia for the 109 clinical samples were 31 to 1058 AFU, 307 to 2843 AFU and 10 to 876 AFU respectively. Membrane antigen data were obtained for all but three samples with severe monocytopenia. PMN-CD64 and MON-CD64 expression shared the same overall trend although relative increases in MON-CD64 expression were higher than PMN-CD64 (*figure 7a*). More detailed examination of individual samples (*table 1*) showed that normal PMN-CD64 expression was only seen with normal (45/52) or intermediate MON-CD64 (7/52), while high PMN-CD64 was usually associated with intermediate (18/22) or high (3/22) MON-CD64. By comparison, MON-Ia expression appeared to be largely independent (p=0.04) of PMN-CD64 (*figure 7b*) although a marked decrease in MON-Ia was always associated with intermediate or high levels of PMN-CD64 (*table 1*). Interestingly, marked decreases in MON-Ia were not seen when MON-CD64 was expressed at high levels.

Patterns of Antigen Expression and Granulocyte Counts:
Evaluated relationships with absolute granulocyte counts suggested (*table 2*) some association with PMN-CD64 expression but none with MON-CD64. There was also an inverse relationship (p<0.0001) between

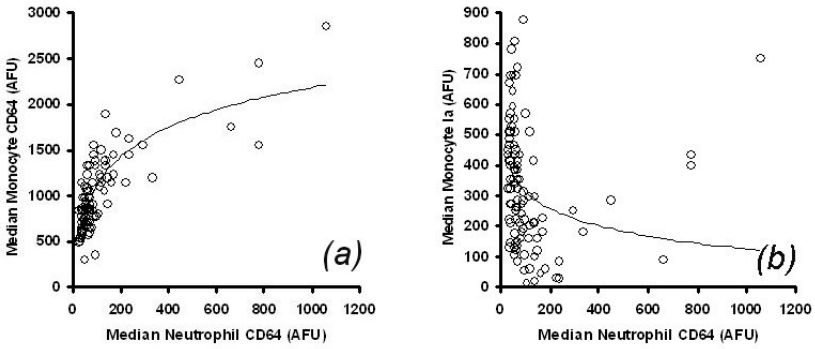

fig 7
Individual sample (n=106) relationships between median neutrophil CD64 and (a) monocyte CD64 and (b) monocyte HLA-DR (Ia). Plotted trendlines are derived from logarithmic plots.

MON-Ia and the absolute granulocyte count with a trend for higher counts with decreasing membrane HLA-DR expression. Examination of other haematological parameters revealed no significant association between neutrophil and monocyte antigen levels and percentages of Immature Granulocytes (data not shown), although samples with high PMN-CD64 were more likely (8/25) to have in excess of 10% band cells compared to samples with normal or intermediate PMN-CD64 (0/84).

Patterns of Antigen Expression and CRP Concentration:
The range of CRP concentrations for the 109 clinical samples analysed in this study was <10 to 460 mg/L. There were 57 samples with a normal CRP (<10 mg/L), 49 between 10 and 100 mg/L, and 3 with concentrations higher than 100 mg/L. When neutrophil and monocyte antigen expression was compared to CRP (*table 3*), high degrees of overall association were observed although individual sample variation was also evident. For example, while high PMN-CD64 and high MON-CD64 expression were always associated with an increased CRP concentration, this could also be seen when PMN-CD64 (11/52) and MON-CD64 (11/65) were normal. Similarly, 11/15 and 9/10 of the patient samples with intermediate or marked decreases in MON-Ia had increased CRP levels. Of note with respect to the analysis of monocytes was that the three samples with severe monocytopenia (and insufficient population events to determine antigen expression) all had CRP concentrations exceeding 200mg/L.

Discussion

The primary aim of this study was to evaluate a two-colour immunofluorescent assay for leukocyte CD64 and HLA-DR membrane

table 1 *Relationships between PMN-CD64 , MON-CD64 and MON-Ia expression for 106 randomly selected clinical samples. Normal, intermediate and high antigen levels as defined in Results. Data excludes three samples with severe monocytopenia.*

	Monocyte CD64		
	Normal	Intermediate	High
Neutrophil CD64			
Normal	45	7	0
Intermediate	19	13	0
High	1	18	3

X^2 statistic, 48.9 (p<0.0001)

	Monocyte Ia		
	Normal	Intermediate Decrease	Marked Decrease
Neutrophil CD64			
Normal	46	6	0
Intermediate	23	5	4
High	12	4	6

X^2 statistic, 15.7 (p=0.04)

	Monocyte Ia		
	Normal	Intermediate Decrease	Marked Decrease
Monocyte CD64			
Normal	57	5	3
Intermediate	21	10	7
High	3	0	0

X^2 statistic, 15.0 (p=0.005)

table 2 *Relationships between neutrophil (CD64) and monocyte (CD64 and Ia) antigen expression and absolute granulocyte counts for 109 randomly selected clinical samples. Ranges for absolute granulocyte counts represent multiples of the upper normal range (8.0 x 10^9/L). Normal, intermediate and high antigen levels as defined in Results; monocyte antigen data excludes three samples with severe monocytopenia.*

	Absolute Granulocyte Count (x 10^9/L)		
	<8.0	8.0 - 16.0	>16.0
Neutrophil CD64			
Normal	42	10	0
Intermediate	17	14	1
High	16	5	4

X^2 statistic, 17.0 (p=0.002)

	Absolute Granulocyte Count (x 10^9/L)		
	<8.0	8.0 - 16.0	>16.0
Monocyte CD64			
Normal	51	13	1
Intermediate	20	14	4
High	2	1	0

X^2 statistic, 9.2 (p=0.06)

	Absolute Granulocyte Count (x 10^9/L)		
	<8.0	8.0 - 16.0	>16.0
Monocyte Ia			
Normal	62	19	0
Intermediate Decrease	10	5	0
Marked Decrease	1	4	5

X^2 statistic, 55.0 (p<0.0001)

markers on a routine haematology analyser with fluorescent capabilities. These two antigens are of particular interest because of the considerable accumulated evidence suggesting that upregulation (neutrophil and monocyte CD64) and downregulation (monocyte HLA-DR) have a

consistent association with sepsis. While further substantiating these associations was not the purpose of this study, our evaluation of antigen expression in 109 randomly-selected samples with clinical requests for CRP did allow us to undertake supplementary studies into patterns of antigen change and their relationships with both CRP concentration and absolute granulocyte count. The value of these additional analyses was to highlight the existence of heterogeneous patterns of antigen expression compared to these widely-used empirical assessments of inflammation/infection.

table 3 *Relationships between neutrophil (CD64) and monocyte (CD64 and Ia) antigen expression and C-Reactive Protein (CRP) concentrations for 109 randomly selected clinical samples. Ranges for CRP concentrations correspond to mild, moderate and severe inflammatory processes as defined by Whicher (18). Normal, intermediate and high antigen levels as defined in Results; monocyte antigen data excludes three samples with severe monocytopenia and CRP levels exceeding 100mg/L.*

	CRP (mg/L)			
	<10	10 - 50	51 - 100	>100
Neutrophil CD64				
Normal	41	8	3	0
Intermediate	16	6	10	0
High	0	2	20	3

X^2 statistic, 62.1 (p<0.0001)

	CRP (mg/L)			
	<10	10 - 50	51 - 100	>100
Monocyte CD64				
Normal	54	8	3	na
Intermediate	3	8	27	na
High	0	0	3	na

X^2 statistic, 67.3 (p<0.0001)

	CRP (mg/L)			
	<10	10 - 50	51 - 100	>100
Monocyte Ia				
Normal	52	11	18	na
Intermediate Decrease	4	4	7	na
Marked Decrease	1	1	8	na

X^2 statistic, 19.8 (p<0.001)

The methodological procedure evaluated in this study was analogous to standard flow cytometry in that EDTA-anticoagulated blood samples were pre-incubated with FITC/PE-labelled monoclonal antibodies, analysed in a laser-illuminated optical system with fluorescent detectors, and the resulting raw-file information processed with cytometry software to determine the nature of specific cell populations. Compared to flow cytometry, the CD4000 analysis is simpler in that instrument calibration and gain setting is continuously maintained for the purposes of routine blood count analysis. Processing of pre-stained samples, subsequent data acquisition (up to 20,000 leukocyte events) and red cell lysis are also part of the automated procedure. There is also no need for washing

and the method has the additional advantage that the simultaneous leukocyte viability measurements [16] provided by the CD4000, minimise potential inconsistencies of antigen measurements that may be associated with leukocyte population deterioration. Data processing using a PC is relatively straightforward and can be achieved in various ways. In this study, downloaded CD4000 List Mode files were converted to standard FCS2.0 format and analysed using WINMDI software. Alternatively, files can be analysed using programmes such as FCS Express v3 (De Novo Software, Thornhill, Ontario, Canada) which open CD4000 files directly without the need for FCS conversion.

Normal ranges for PMN-CD64, MON-CD64 and MON-Ia were defined by analysis of 25 normal adults and the CD4000 method was shown to have good duplicate precision and agreement with a flow cytometry procedure. Some day to day variation was however noted in some individuals but the observed quantitative differences were of minor interpretative significance. One potentially important observation however was that storage of EDTA-anticoagulated samples for more than 6 hours at room temperature could lead to increases in apparent PMN-CD64 expression. Although there was significant individual sample variation, there was a mean +16% increase in antigen staining at 12 hours compared to that obtained 1 hour after venesection. As there was no evidence that the observed increased expression was associated with changes in leukocyte viability, we conclude that this could be due to low-level *in vitro* activation or progressive exposure of cryptic CD64 antigens during the early stages of leukocyte biological deterioration.

Membrane CD64/HLA-DR antigen levels were semi-quantitatively expressed as arbitrary fluorescent units (AFU) in a similar way to many previous research and validation studies. This approach was taken as the alternative practice of reporting antigen expression as percentages of cells exceeding a defined fluorescence threshold are limited. This is illustrated in figure 8 which shows PMN-CD64 results of the 109 clinical samples as both AFU and percentages $CD64^+$ positive neutrophils (where the discrimination threshold was set at a level corresponding to the upper 95% distribution of 10 normal control samples). While there is a very good overall correlation, the method of determining AFU is more informative in terms of detecting differences at very low and very high levels of expression and could be important for serial patient monitoring.

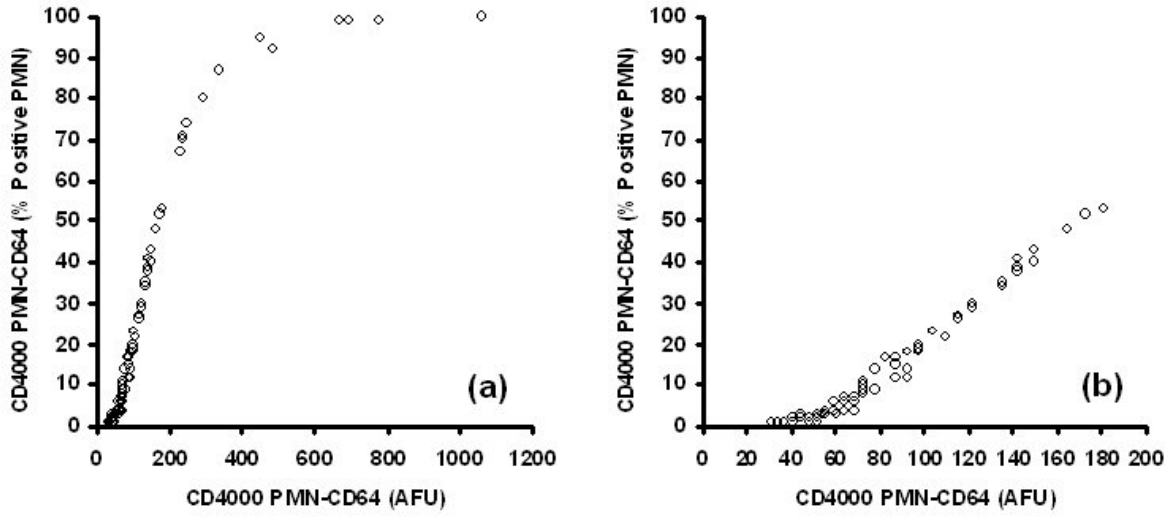

fig 8
Comparison of alternative reporting methods for PMN-CD64 expression. The 109 clinical samples analysed in this study were processed with the CD4000 to determine both the quantitative level of antigen expression (AFU) and the percentages of CD64$^+$ neutrophils where the fluorescence threshold used to discriminate between negative and positive was set at the upper 95% percentile of PMN-CD64 expression by 10 normal samples. Plot (a) shows the comparison for all samples while plot (b) shows the comparison for samples with lower PMN-CD64 expression of <200 AFU.

While the CD4000 method of antigen quantitation described in this study was considered adequate in terms of preliminary method design, and for making relative comparisons of antigen density, we accept that this is not ideal with respect to the need for standardising inter-institutional reporting practices. In this context, improvements in data standardisation can be achieved by quantifying antigen molecules per cell[6, 19] or by reference to internal fluoresceinated control (calibration) particles[20, 21]. With specific respect to neutrophil CD64 measurements, this latter principle is utilised by the Leuko64^{TM} assay kit (Trillium Diagnostics LLC, Maine USA). This approach goes some way to ensuring consistency of antibody reagent source and fluorochrome-protein characteristics of monoclonal antibody conjugates but it is important to note that fluoresceinated particles do not confirm whether or not leukocytes are optimally stained.

As indicated earlier, there is considerable support and clinical evidence for using measurements of neutrophil CD64 expression in assessing patients with suspected sepsis[1-8]. *In vitro* observations further substantiate this contention in that upregulated expression of PMN-CD64 is mediated by lipopolysaccharides[22], γ-interferon[23] and G-CSF[24]. In contrast to a number of other potential leukocyte markers for sepsis, PMN-CD64 has a number of potential practical advantages in that it is

expressed at very low level by normal neutrophils and is relatively insensitive to sample manipulation[2, 25]. Similarly, the measurement of monocyte HLA-DR expression has also been widely evaluated and shown to be significantly decreased in sepsis[12, 14, 26] and associated with transient immunosuppression[13]. The combined analysis of CD64 and HLA-DR, two markers which show opposite trends of upregulation and downregulation in sepsis, may be advantageous compared to single antigen assessments and a supplementary aim of this study was therefore to examine antigen pattern heterogeneity in a series of 109 patients in relation to two commonly used indicators (CRP and absolute granulocyte count) of inflammation/infection. As such, this study is the first to specifically assess relationships between PMN-CD64, MONM-CD64 and MON-Ia for individual samples.

The results showed that while neutrophil and monocyte CD64 upregulation appeared to follow a similar trend, relationships with MON-Ia expression were more complex. Significant reductions in MON-Ia are characteristically seen in early sepsis, with progressive reattainment of normality as sepsis-associated transient immunosuppression is overcome[25], and our observations showed that while markedly depressed monocyte HLA-DR expression was associated with high PMN-CD64 this was in contrast to MON-CD64 which was either normal or only moderately increased. The possibility that these different trends were to some extent a reflection of differences in cell population kinetics, where the half-life of neutrophils (6-9 hours) is considerably shorter than monocytes (8-72 hours), and differential cellular responses to inflammation/infection is supported by a number of further observations. For example, all five patients with absolute granulocyte counts exceeding 16.0 x 10^9/L showed markedly reduced MON-Ia expression. Four of these showed a consistent pattern of high PMN-CD64, intermediate MON-CD64 and high (>100mg/L) CRP concentrations. In contrast, the fifth patient showed an intermediate increase in PMN-CD64, normal MON-CD64 and a normal CRP. Interestingly, this particular patient who was being artificially respirated and had a long history of intermittent septic episodes showed a significant increase in CRP in the immediate period following antigen testing. An additional finding was that the samples with profound monocytopenia all had very high PMN-CD64 expression and CRP concentrations.

In summary, this study has demonstrated the design feasibility of a fluorescent method for the semi-quantitative determination of neutrophil (CD64) and monocyte (CD64 and HLA-DR) antigens associated with the assessment of sepsis. The combined use of CD64 and HLA-DR is

considered more informative than using CD64 alone, and while standardisation of the process could be improved by method modifications with respect to fluorescent quantitation, the procedure nevertheless remains simple and relatively straightforward to implement on analysers with fluorescence detection capability. We believe that the procedures described in this communication could overcome a number of limitations associated with flow cytometry and provide a potential indicator of sepsis at relatively low cost and with a minimum of technical expertise.

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References

1 Guyre PM, Campbell AS, Kniffin WD, Fanger MW. Monocytes and polmorphonuclear neutrophils of patients with streptococcal pharyngitis express increased numbers of type I IgG Fc receptors. J Clin Invest 1990;86:1892-1896
2 Davis BH, Bigelow NC, Curnutte JT, Ornvold K. Neutrophil CD64 expression. Potential diagnostic indicator of acute inflammation and therapeutic monitor of interferon-γ therapy. Lab Hematol 1995;1:3-12
3 Hirsh M, Mahamid E, Bashenko Y, Hirsh I, Krausz MM. Overexpression of the high-affinity Fcgamma receptor (CD64) associated with leukocyte dysfunction in sepsis. Shock 2001;16:102-108
4 Naccasha N, Gervasi MT, Chaiworapongsa T, Berman S, Yoon BH, Maymon E, Romero R. Phenotypic and metabolic characteristics of monocytes and granulocytes in normal pregnancy and maternal infection. Am J Obstet Gynecol 2001;185:1118-1123
5 Layseca-Espinosa E, Perez-Gonzales LF, Torres-Montes A, Baranda L, de la Fuente H, Rosenstein Y, Gonzalez-Amaro R. Expression of CD64 as a potential marker of neonatal sepsis. Pediatr Allergy Immunol 2002;13:319-327
6 Ng PC, Li G, Chui KM, Chu WC, Li K, Wong RP, Chik KW, Wong E, Fok TF, 2004. Neutrophil CD64 is a sensitive diagnostic marker for early onset neonatal infection. Pediatr Res 2004;56: 796-803
7 Fjaertoft G, Hakansson L, Ewald U, Foucard T, Venge P. Neutrophils from term and preterm newborn infants express high affinity Fc-gamma-receptor I (CD64) during bacterial infections. Pediatr Res 1999;45:871-876
8 Allen E, Bakke AC, Purtzer MZ, Deodhar A. Neutrophil CD64 expression: Distinguishing acute inflammatory autoimmune disease from systemic infections. Ann Rheum Dis 2002;61:522-525
9 Crocker PJ, Quick G, McCombs W. Occult bacteremia in the emergency department: diagnostic criteria for the young febrile child. Ann Emerg Med 1985;14:1172-1177
10 Werman HA, Brown CG. White blood cell count and differential count. Emerg Med Clin North Am 1986;4:41-58

11 Shapiro MF, Greenfield S. The complete blood count and leukocyte differential count. An approach to their rational application. Ann Intern Med 1987;106:65-74
12 Muller-Kobold AC, Tulleken JE, Zijlstra JG, Sluiter W, Hermans J, Kallenberg CG, Tervaert J. Leukocyte activation in sepsis; correlations with disease state and mortality. Intensive Care Med. 2000;26:883-892
13 Le Tulzo Y, Pangault C, Amiot L, Guilloux V, Arvieux C, Camus C, Fauchet R, Thomas R, Dernous B. Monocyte human leukocyte antigen-DR transciptional downregulation by cortisol during septic shock. Am J Respir Crit Care Med 2004;169:1144-1151
14 Sedlackova L, Prucha M, Dostal M. Immunological monitoring of sepsis using flow cytometry – quantitation of monocyte HLA-DR expression and granulocyte CD64 expression. Epidemiol Mikrobiol Immunol 2005;4:54-61
15 Marshall P, Hung D, Yuan J, Kim YR. Rapid, automated, closed-tube quantitation of CD4+ and CD8+ T-cell populations on the Cell-Dyn 4000 hematology analyzer. Lab. Hematol. 2000;3:137-143
16 Erwa W, Bauer FR, Etschmaier R, Steiner U, Scott CS, Sedlmayr P. Analysis of aged samples with the Abbott CD4000 hematology analyzer. Eur J Lab Med 1998;6:4-15
17 Passing H, Bablok W. A new biometrical procedure for testing the equality of measurements from two different analytical methods. J Clin Chem Biochem 1983;21:709-720
18 Whicher J. (1998) C-reactive protein (CRP). In ‘Clinical Laboratory Diagnostics‘. Ed: L Thomas. T-H Books, Frankfurt. pp 700-706
19 Poncelet P. Microbeads and flow cytometry: how and why put the "-metry" in immuno-cytometry? Ann Biol Clin. 2004;62:53-57
20 Gratama JW, D’Hautcourt JL, Mandy F, Rothe G, Barnett D, Janossy G, Papa S, Schmitz G, Lenkei R. Flow cytometric quantitation of immunofluorescence intensity: problems and perspectives. European Working Group on Clinical Cell Analysis. Cytometry 1998;33:166-178
21 Vogt RF, Whitfield WE, Henderson LO, Hannon WH. Fluorescence intensity calibration for immunophenotyping by flow cytometry. Methods 2000;21:289-296
22 Wagner C, Deppisch R, Denefleh B, Hug F, Andrassy K, Hansch GM. Expression patterns of the lipopolysaccharide receptor CD14 on the Fcgamma receptors CD16 and CD64 on polymorphonuclear neutrophils: Data from patients with severe bacterial infection and lipopolysaccharide-exposed cells. Shock 2003;19:5-12
23 Schiff DE, Rae J, Martin TR, Davis BH, Curnutte JT. Increased phagocyte Fc gammaR1 expression and improved Fc gamma-receptor-mediated phagocytosis after in vivo recombinant human interferon-gamma treatment of normal human subjects. Blood 1997;90:3187-3194
24 Kakinoki Y, Kubota H, Yamamoto Y. CD64 surface expression on neutrophils and monocytes is significantly upregulated after stimulation with granulocyte-stimulating factor during CHOP chemotherapy for patients with non-Hodgkins lymphoma. Int J Hematol 2004;79:55-62
25 Davis B. Quantitative neutrophil CD64 expression: Promising diagnostic indicator of acute inflammation or systemic acute inflammatory response. Clin Immunol Newsletter 1996;6:121

26 Drossou-Agakidou V, Kanakoudi-Tsakalidou F, Sarafidis K, Tzimouli V, Parakou A, Kremenopoulos G, Germenis A. In vivo effect of rhGM-CSF and rhG-CSF on monocyte HLA-DR expression of septic neonates. Cytokine 2002;18:260-265

Chapter 9

Haematological indices, sepsis markers (crp, tnf-α, ifn-γ, il-6 and il-10) and neutrophil cd64 expression: comparative trends during experimental human endotoxemia

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submitted

Abstract

Background

CD64 is a high affinity neutrophil CD64 FcγRI receptor expressed by activated neutrophils that has been recently evaluated as a potential sepsis parameter. In the present study, the kinetics of neutrophil membrane CD64 expression was examined during a standardized inflammatory response, using a human endotoxemia model, and compared with haematological indices, CRP, cytokines and interleukins.

Methods

Ten healthy subjects received 2 ng/kg intravenous E. coli lipopolysaccharide (LPS). Neutrophil CD64 expression was measured and quantitated with a routine haematology analyser (Abbott Cell Dyn 4000) using supplementary software. Haematological indices and CRP were measured with standard laboratory methods, and cytokines determined with simultaneous Luminex Assays.

Results

After the administration of LPS, neutrophil CD64 showed a biphasic response. The first increase was seen after 1 hour, while the second increase started after 6 hours and reached its maximum at 22 hours. For the absolute neutrophil count and the immature granulocytes a rapid decline was observed followed by an increase with a peak value at 12 hours. CRP concentrations showed a slow response starting at 6 hours and reaching their highest levels 22 hrs after the administration of LPS. The cytokines and interleukins reached their maximum response within 1-2 hours. The maximum values of pro-inflammatory cytokines correlated with the CD64 expression at 22 hours after LPS administration, whereas this correlation was not found for the anti-inflammatory IL-10.

Conclusion

During experimental human endotoxemia, neutrophil CD64 expression shows a biphasic response. The correlation between the early release of pro-inflammatory cytokines and CD64 expression suggests that CD64 expression is a quantitative marker of innate immunity.

Introduction

The annual incidence of sepsis in the United States is 50-95 cases per 100,000 with approximately 9% being categorized as severe and 3% complicated by septic shock[1]. As mortality increases with progressive sepsis, its early detection is very important. One of the central features of the host response to bacterial infection is the recruitment and activation of neutrophils, typically manifested by blood neutrophilia. Other haematological indicators of infection include the morphological presence of neutrophils with toxic changes (e.g. hypergranulation and

Dohle bodies), increased proportions of non-segmented or band neutrophils and circulating immature granulocytes (metamyelocytes and myelocytes)[2]. However, the non-specific range of diverse conditions associated with changes in neutrophil counts[3], as well as the frequent occurrence of mild to moderate neutropenia in the acute phase of infection and the statistical and morphological limitations of band cell enumeration[4-6], considerably limits their diagnostic specificity.
In addition to haematological investigations, a number of biochemical and immunological analyses are variously used as diagnostic aids. These include acute phase proteins such as c-reactive protein (CRP) and procalcitonin (PCT), cytokines such as tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ), and interleukins such as IL-6 and IL-10[7-12]. While many of these have been used to indicate disease severity[13-15] and to supplement full blood count (FBC) screening in the assessment of suspected infection, they are less widely available and are also affected by confounding factors.
We recently described a practical procedure for quantitative fluorescent neutrophil CD64 (nCD64) measurements with a haematology analyser[16]. nCD64 corresponds to the high-affinity FcγRI receptor expressed by monocytes but not by normal neutrophils. Upregulated and detectable nCD64 is characteristically seen in patients with infection and its measurement is gaining acceptance as a useful marker in the assessment of sepsis[17-22]. Relatively little is known however about changes in nCD64 during the early stages of inflammation. To investigate this particular question, the present study examined the modulation of nCD64 expression during a standardized inflammatory stimulus using experimental human endotoxemia and compared this to changes in haematological and non-haematological (CRP, IFN-γ, TNF-α, IL-6 and IL10) parameters. Furthermore, the cytokine response during endotoxemia was correlated with CD64 expression to examine whether quantitative changes in CD64 expression reflect the magnitude of the innate immunity response**.**

Materials and Methods

Subjects:

This study was approved by the local ethics committee and informed consent was obtained from each volunteer. Prior to the single-dose intravenous administration of 2 ng/kg E. coli O:113 LPS (US Pharmcopia, Rockvilee, MD, USA), ten healthy subjects (4 male, 6 female; mean age 21 years, range 18-24 years) entered into the study were checked by routine medical examination, electrocardiography and blood analysis. With the exception of oral contraceptives, none of the

volunteers used prescription medication, aspirin or other non-steroid anti-inflammatory drugs and all were HIV and Hepatitis B negative. Medical appraisal confirmed that there was no history of a febrile illness in the 2 weeks preceding the study, and in the 12 hour period prior to LPS administration all subjects refrained from caffeine, alcohol and food. In the post-LPS period, vital signs were continuously monitored and at various time intervals EDTA and heparin anticoagulated blood samples were taken for haematological, nCD64, CRP, interleukins and cytokines measurements.

Analysis of Neutrophil CD64 (nCD64) Expression:
nCD64 expression was measured at baseline and 1, 2, 4, 6, 12 and 22 hours after LPS administration. As previously described[16], the immunofluorescent detection and quantification of nCD64 was performed using the Cell-Dyn CD4000 haematology analyser (Abbott Diagnostics, Santa Clara, CA, USA). In brief, non-anticoagulated vacutainer tubes containing 100 µL whole blood plus 20 µL anti-CD64 (FITC; Becton Dickinson) and 15 µL anti-Ia (PE; Becton Dickinson), were processed using the CD4000 automated CD4/CD8 assay mode after preliminary incubation at room temperature for 10 minutes. Raw data files were downloaded to a PC for population analysis. Primary gating of the neutrophil population was facilitated with WINMDI software (http://facs.scripps.edu/software.html), using Ia expression to exclude monocytic components and optical characteristics (0^o versus 7^o) to exclude lymphocytes. This was followed by post-acquisition fluorescent channel compensation using WINList 4.0 software (Becton Dickinson). Statistical analyses of neutrophil FL1 histograms (3-decade log) were then undertaken to obtain median nCD64 staining intensities quantitatively expressed as Arbitrary Fluorescent Units (AFU). In a previous study the normal range for nCD64 was determined at a mean$\pm$SD of 94$\pm$14 AFU[16].

Haematological Studies:
White blood cell (WBC) and absolute neutrophil counts (ANC) were measured at baseline and 1, 2, 4, 6, 12 and 22 hours after LPS administration using a Sysmex XE-2100 (Toa, Kobe, Japan) analyser. Immature granulocytes were enumerated with Sysmex instrument software (XE-master), while band cell counts were obtained by conventional microscopy (2x 200 cells) of May-Grünwald Giemsa stained blood smears.

Analysis of CRP, TNF-α, IFN-γ, IL-6 and IL-10:
Serum CRP was measured at baseline and 1, 2, 4, 6, 12 and 22 hours after LPS administration using a turbidimetric method (Aeroset, Abbott Laboratories, Abbott Park, IL, USA). Concentrations of tissue necrosis factor (TNF-α), γ-interferon (IFN-γ), and interleukins 6 (IL-6) and 10 (IL-10) were determined at baseline and 1, 1½, 2, 3 and 4 hours after LPS administration using the simultaneous Luminex Assay (R&D Systems, Minneapolis MN, USA)[23].

Statistical Analysis:
Descriptive results of continuous variables were expressed as mean ± standard error of the mean (SEM). Data analysis was performed using ANOVA with repeated measures. A *p*-value <0.05 was considered significant. Individual peak values of cytokines and interleukins were correlated (Pearson) with neutrophil CD64 expression at 22 hours after LPS administration.

Results
Subjects and Pre-LPS Baseline Analyses:
Table 1 shows baseline values of the measured parameters.

Post-LPS – Neutrophil CD64 (nCD64) Expression:
The nCD64 expression following LPS administration shows a biphasic increment (*figure 1*). A first rise was seen after one hour with a maximum of 133±6 AFU after 2 hours (*p*=0.05), the second increment started at 12 hours with a maximum of 167±13 AFU at 22 hours (*p*=<0.0001).

Post-LPS – Haematological Parameters:
A rapid initial decline in the ANC following the intravenous administration of LPS was observed (*figure 2 and table 1*). At 1 hour, the mean ANC was $1.0\pm0.1x10^9$/L compared to a pre-LPS value of $4.9\pm1.2x10^9$/L (*p*<0.0001). Thereafter, the ANC showed a progressive increase to $11.5\pm0.7x10^9$/L at 6 hours and a subsequent declining trend to $6.6\pm1.0x10^9$/L at 22 hours (*p*<0.0001 for the whole curve). The immature granulocyte count showed a similar overall trend (*p*=0.03), although all values remained within the normal range (*figure 2 and table 1*). Band cells showed a clearer increase to maximal values 2 to 6 hours post-LPS (*figure 2 and table 1*). Mean band cell counts during this transient increase was 15±3%. Subsequent progressive declines to normal (<5%) band cell values were seen for all subjects at 22 hours (*p*<0.0001). Changes in ANC and band cell counts were not accompanied by any observable increase in neutrophil toxic granulation.

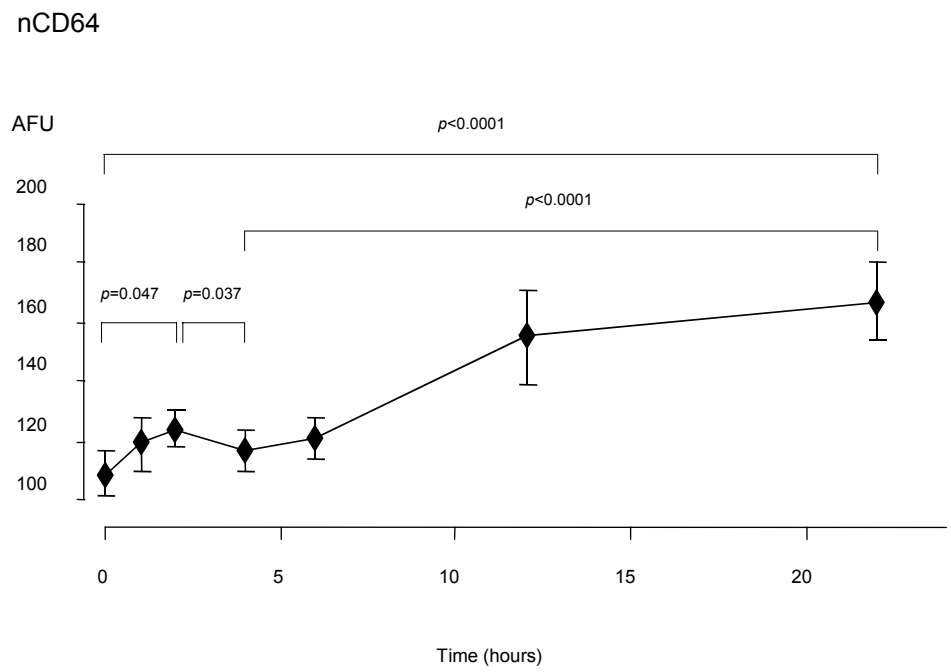

fig 1
Time course trends of neutrophil CD64 (nCD64) following experimental LPS administration.

Post-LPS – CRP, TNF-α, IFN-γ, IL-6 and IL-10:
Serum CRP concentrations showed a relatively slow response compared to changes in haematological parameters and nCD64 expression. The mean CRP concentration at 6 hours was 6.9±0.6 mg/L, which then progressively increased over the post-LPS period to mean concentrations at 12 and 22 hours of 24.7±1.1 mg/L and 39.7±4.9 mg/L respectively (p<0.0001, *figure 2 and table 1*).
For comparison, TNF-α and IFN-γ increased within one hour of LPS administration to mean peak values at 90 minutes of 1047±285 pg/mL and 170±42 pg/mL respectively (both p<0.0001, *figure 3 and table 1*). Decreases to relatively normal levels were seen within 4 hours post-LPS. For the interleukin markers, IL-6 increased within one hour to a mean peak value of 8338±4560 pg/mL at four hours (*figure 3 and table1*) (p=0.009), while IL-10 increased within one hour to a peak value of 112±26 pg/mL at two hours (*figure 3 and table 1*) (p<0.0001). The nCD64 expression at 22 hours after LPS administration is associated with peak values of TNF-α (R^2=0.76, p=0.002), IFN-γ (R^2=0.78, p=0.002) and IL-6 (R^2=0.81, p=0.001), whereas this correlation was not found for IL-10 (R^2=0.05, p=0.54). Also, there was no significant correlation between nCD64 expression and CRP (R^2=0.16, p=0.28).

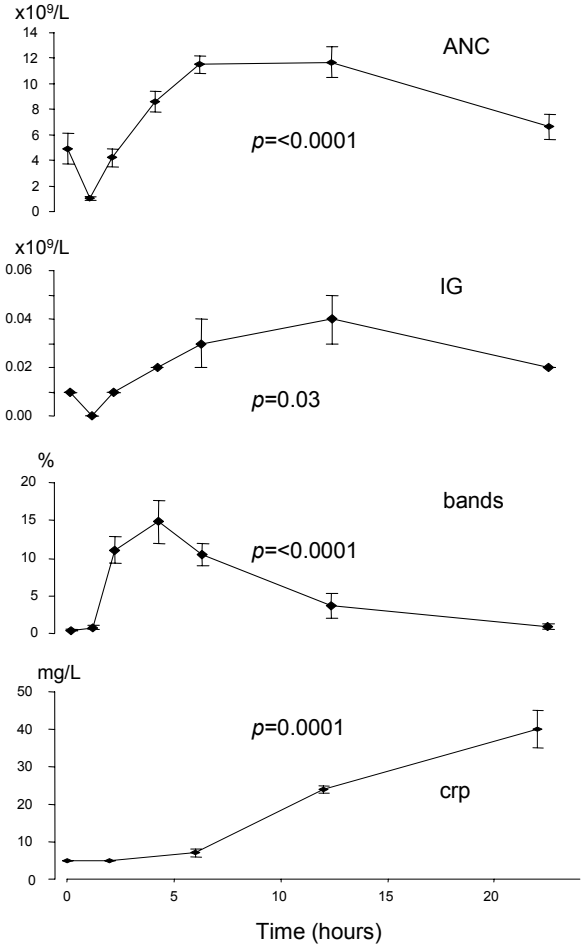

fig 2
Time course trends of sepsis parameters following experimental LPS administration; absolute neutrophil count (ANC, x10^9/L), absolute immature granulocyte count (IG, x10^9/L), microscopic band count (%) and C-reactive protein (CRP, mg/L)

table 1 Comparison of all measured parameters at baseline level and time to reach maximum value

parameter	Reference value	Mean baseline value±SEM	Mean maximum value± SEM	Time to mean maximum value (h)	p-value
ANC (x10^9/L)	2.0 - 6.5	4.9±1.2	11.6±1.2	6-12	<0.0001
Band Cells (%)	<5	<5±0	15±3	4-6	<0.0001
IG (x10^9/L)	0.05	0.01±0	0.04±0.01	6-12	0.03
CRP (mg/L)	<5	7.2±2.4	41±5	22	0.0001
IFN-γ (pg/mL)	nd	10.4±2.6	170±42	1.5	<0.0001
TNF-α (pg/mL)	nd	8±0	1047±285	1.5	<0.0001
IL-10 (pg/mL)	nd	8±0	112±26	2	<0.0001
IL-6 (pg/mL)	nd	11.7±2.7	8338±4560	4	0.009
nCD64[a] (AFU)	<145	108.8±7.5	133±6	1-2	0.047
			264±13	12-22	<0.0001

[a] Neutrophil membrane CD64 expression expressed as Arbitrary Fluorescent Units (AFU). nd = not detectable

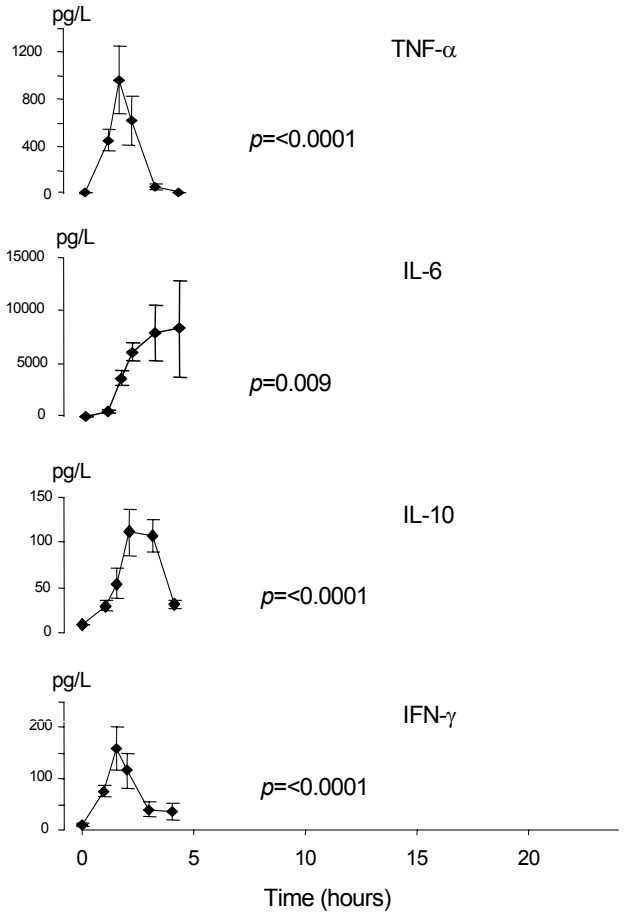

fig 3
Time course trends of sepsis parameters following experimental LPS administration; tumour necrosis factor (TNF-α, pg/mL), interleukin-6 (IL-6, pg/mL), interleukin-10 (IL-10, pg/mL) and γ-interferon (IFN-γ, pg/mL)

Discussion

The present study demonstrates that in a standardized inflammation model nCD64 expression shows a biphasic response. The first phase of this response coincides with the increment of cytokines, whereas the second phase shows the same trend as the CRP. The nCD64 expression at 22 hours after LPS administration is associated with peak values of pro-inflammatory cytokines, whereas this correlation was not found for IL-10 and CRP, suggesting that the nCD64 expression is a quantitative marker of the innate immune response. Furthermore, this study confirms that nCD64 can be rapidly and easily measured using a routine haematology analyser.

Upregulation and increased expression of nCD64 appears to be a sensitive marker for early-onset clinical infection in newborn children[22, 24]. In adults its use has been variably suggested for differentiating systemic infection from active inflammatory disease[25], monitoring γ-interferon therapy[18] and as an indicator for initiating or discontinuing

antibiotic treatment[22]. nCD64 analysis may also be useful for assessing sepsis while haematological parameters such as leukocyte/granulocyte counts, or the presence of immature granulocytes and band cells are relatively uninformative[26, 27]. Especially in young children and elderly patients, disorders with primary disturbances in neutrophil numbers, and conditions where neutrophil counts are affected by myelosuppressive therapies, nCD64 analysis might be helpful. Our observation that nCD64 expression correlates with a rise in pro-inflammatory cytokines 20 hours earlier further substantiates the notion that nCD64 expression is a quantitative measure of the innate immune response (*table 2*).

table 2 Correlation between CD64, 22 hours after LPS administration and other sepsis parameters at their peak value

parameter	R^2	*p*-value
CRP	0.1666	0.2755
IFN-γ	0.7812	0.0016
TNF-α	0.7614	0.0021
IL-10	0.0548	0.5444
IL-6	0.8091	0.0010

The potential value of nCD64 measurements in patients with suspected sepsis has been described previously. A sensitivity of 94.1% and a specificity of 84.9%, and positive predictive likelihood ratio of 6.24 have been reported[28]. Relatively little is known about the changes in the expression of this receptor during the initial stages of infection in humans. In order to investigate this particular point, we used an accepted model for Gram-negative sepsis (experimental human endotoxemia) and examined the resulting trends of nCD64 expression and compared these with a number of other parameters (i.e. CRP, ANC, IG, band cells, IFN-γ, TNF-α, IL-6, and IL-10) used for monitoring inflammatory processes. Human LPS exposure has been used previously by other investigators as an experimental model to examine diverse aspects of the inflammatory response including vascular permeability[29], neuropsychological functions[30], pharmacomodulation[31, 32], and changes in leukocyte antigen expression[33-35].

In this study, we show that changes in nCD64 expression following LPS administration can generally be characterized as biphasic. There was an initial increased expression within 1-2 hours while a second, more significant incremental nCD64 response, starting at 6 hours and reaching maximum levels at 12-22 hours, was seen in all subjects. The mean peak values for these two phases were 120 and 165 AFU, respectively, compared to a mean baseline level of 102 AFU and a previously defined upper normal limit of 109 AFU. In our experimental LPS model, nCD64

increments were above the upper normal limit, but less pronounced compared to septic patients[16].
This almost certainly reflects the relatively mild and limited exposure to endotoxin in an experimental setting compared to clinically important sepsis where endotoxin exposure is more prolonged and typically of greater magnitude.

In examining relationships between haematological changes and nCD64 expression, we found that LPS administration was accompanied by an initial rapid decline in the absolute neutrophil (ANC) and immature granulocyte (IG) counts at 1 hour, followed by an increase that reached a maximum at 6 hours and then a decline to relative normality at 22 hours. The possibility that the initial ANC decline was a result of increased sequestration (extravasculation), with the subsequent increase resulting from an expanded release of marrow neutrophils, is supported to some extent by the observation of transiently increased proportions of band cells between two and six hours post-LPS. Moreover, the automated IG count showed also a small but significant increase in the same period although values remained within the normal range. We hypothesize that the initial small increase in nCD64 expression that was seen and the more consistency observed decline in the ANC might be due to a direct modulating effect of LPS on circulating mature neutrophils. For the second and more sustained increase in nCD64 expression however, which was paralleled by a rising ANC, we postulate that newly released neutrophils from marrow/storage pools may have constitutively upregulated nCD64 levels.

Analysis of the biochemical and immunological markers revealed relatively little change in CRP levels in the first six hours post-LPS, although a consistent and significant progressive increase was seen thereafter. In contrast, TNF-α, IFN-γ and IL-10 increased significantly within one hour and IL-6 within 2 hours. These findings are in agreement with previous studies[29].
While interpreting the results of this study, it is important to note that the LPS administration comprised a single dose and that any subsequent effects would essentially be short-lived as the stimulus is progressively reduced by the host response (LPS clearance). Nevertheless, while a single dose exposure cannot be extrapolated to clinical situations where stimulation by a mediating agent such as LPS is likely to be more prolonged, our study provide valuable insights into the relative rates and magnitudes of early cellular and biochemical changes. By this means, we found that changes in nCD64 expression proceeded CRP but lagged behind increases in TNF-α, IFN-γ, band cells, IL-6 and IL-10. With

regards to laboratory and clinical application, the cytokine and interleukin assays are unlikely to be available in many routine laboratories while the more widely available band cell estimates and CRP measurements both suffer from low diagnostic specificity[36].
In conclusion, compared to other haematological indices nCD64 expression shows a biphasic response to LPS administration. A correlation of nCD64 is found with TNF-α, IFN-γ and IL-6, but not with IL-10 and CRP (*table 2*). Measurements of interleukins and cytokines takes some time thereby hampering the availability of these markers in sepsis diagnosis. Therefore nCD64 might be a potential sepsis marker, which can be measured on a routine haematology analyser.

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References

1 Annane D, Bellissant E, Cavaillon JM. Septic shock. Lancet 2005;365:63-78
2 Ardron MJ, Westengard JC, Dutcher TF. Band neutrophil counts are unnecessary for the diagnosis of infection in patients with normal total leukocyte counts. Am J Clin Pathol 1994;102:646-649
3 Bain BJ. Blood cells: a practical guide. 3ed. Oxford, Blackwell Sciences 2002:205pp
4 Cornbleet PJ. Clinical utility of the band cell count. Clin Lab Med 2002;1:101-136
5 Van der Meer W, de Keijzer MH, Scott CS. Automated flagging influencing the inconsistency and bias of band cell and atypical lymphocyte morphological differentials. Clin Chem Lab Med 2004;4:371-377
6 Van der Meer W, van Gelder W, de Keijzer R, Willems JL. Does the band cell survive the 21st century? Eur J Haematol 2006;67:251-254
7 Balcl C, Sungurtekin H, Gurses E, Sungurtekin U, Kaptanoglu B. Usefulness of procalcitonin for diagnosis of sepsis in the intensive care unit. Crit Care 2003;7:85-90
8 Martins GA, Da Gloris Da Costa Carvalho M, Rocha Gattass C. Sepsis: a follow-up of cytokines production in different phases of septic patients. Int J Mol Med 2003;11:585-591
9 Hengst JM. The role of C-reactive protein in the evaluation and management of infants with suspected sepsis. Adv Neonatal Care. 2003;3:3-13
10 Carrigan AD, Scott G, Tabrizian M. Toward resolving the challenge of sepsis diagnosis. Clin Chem 2004;50:1301-1314
11 Aikawa N, Fujishama S, Endo S, Sekine I, Kogawa K, Yamamoto Y et al. Multicenter prospective study of procalcitonin as an indicator of sepsis. J Infect Chemother 2005;3:152-159

12 Robertshaw HJ, Brennan FM. Release of tumour necrosis factor alpha (TNFalpha) by TNFalpha cleaving enzyme (TACE) in response to septic stimuli in vitro. Br J Anaesth 2005;94:222-228
13 Hatherill M, Tibby SM, Turner C, Ratnavel N, Murdoch IA. Procalcitonin and cytokine levels: relationship to organ failure and mortality in pediatric septic shock. Crit Care Med. 2000;28:2591-2594
14 Heagy W, Nieman K, Hansen C, Cohen M, Danielson D, & West MA. Lower levels of whole blood LPS-stimulated cytokinee release are associated with poorer clinical outcomes in surgical ICU patients. Surg Infect 2003;4:171-180
15 Castelli PC, Pognani C, Meisner M, Stuani A, Bellomi D, Laura Sgarbi L. Procalcitonin and C-reactive protein during systemic inflammatory response syndrome, sepsis and organ dysfunction. Critical Care 2004;8:234-242
16 Van der Meer W, van Dun L, Klein Gunnewiek J, Scott CS. Simultaneous determination of membrane CD64 and HLA-DR expression by blood neutrophils and monocytes using the monoclonal antibody fluorescence capability of a routine haematology analyser. J Immunol Method. *In press*
17 Guyre PM, Campbell AS, Kniffin WD, & Fanger MW. Monocytes and polymorphonuclear neutrophils of patients with streptococcal pharyngitis express increased numbers of type I IgG Fc receptors. J Clin Invest 1990;86:1892-1986
18 Davis BH, Bigelow NC, Curnutte JT, Ornvold K. Neutrophil CD64 expression. Potential diagnostic indicator of acute inflammation and therapeutic monitor of interferon-γ therapy. Lab Hematol 1995;1:3-12
19 Hintz KA, Rasias AJ, Wardwell K, Moss ML, Morganelli PM, Pioli PA et al. (2002) LPS induces rapid metalloprotease-mediated shedding followed by up-regulation of the monocyte hemoglobin scavenger receptor CD163. J Leukoc Biol 2002;72:711-717
20 Naccasha N, Gervasi MT, Chaiorapongsa T, Berman S, Yoon BH, Maymon E, Romero R. Phenotypic and metabolic characteristics of monocytes and granulocytes in normal pregnancy and maternal infection. Am J Obstet Gynecol 2001;185:1118-1123
21 Layseca-Espinosa E, Perez-Gonzales LF, Torres-Montes A, Baranda la Fuente H, Rosenstein Y, Gonzalez-Amaro R. Expression of CD64 as a potential marker of neonatal sepsis. Pediatr Allergy Immunol 2002;13:319-327
22 Ng PC, Li G, Chui KM, Chu WC, Li K, Wong RP et al. Neutrophil CD64 is a sensitive diagnostic marker for early onset neonatal infection. Pediatr Res 2004;56:796-803
23 Prabhakar U, Eirikis E, Davis HM. Simultaneous quantification of proinflammatory cytokines in human plasma using the LabMAP™ assay. J Immunol Methods 2002;260:207-218
24 Fjaertoft G, Hakansson L, Ewald U, Foucard T, Venge P. Neutrophils from term and preterm newborn infants express high affinity Fc-gamma-receptor I (CD64) during bacterial infections. Pediatr Res 1999;45:871-876
25 Allen E, Bakke AC, Purtzer MZ, Deodhar A. Neutrophil CD64 expression: Distinguishing acute inflammatory autoimmune disease from systemic infections. Ann Rheum Dis 2002;61:522-525
26 Crocker PJ, Quick G, McCombs W. Occult bacteremia in the emergency department: diagnostic criteria for the young febrile child. Ann Emerg Med 1985;14:1172-1177

27 Shapiro MF, Greenfield S. The complete blood count and leukocyte differential count. An approach to their rational application. Ann Intern Med 1987;106:65-74
28 Davis BH, Bigelow NC. Comparison of neutrophil CD64 expression, manual myeloid immaturity counts, and automated hematology analyzer flags as indicators of infection or sepsis. Lab Hematol 2005;2:137-147
29 Van Eijk LT, Pickkers P, Smits P, van den Brock W, Bouw MP, van der Hoeven JG. Microvascular permeability during experimental human endotoxemia: an open intervention study. Crit Care 2005;9:157-164
30 Krabbe KS, Reichemberg A, Yirmiya R, Smed A, Pedersen BK, & Bruunsgaard H. Low-dose endotoxemia and human neuropsychological functions. Brain Behav Immun 2005;19:453-60
31 Bunnell E, Lynn M, Habet K, Neumann A, Perdomo CA, Friedhoff LT et al. A lipid A analog, E5531, blocks the LPS response in human volunteers with experimental endotoxemia. Crit Care Med 2000;28:2713-2720
32 Pleiner J, Schaller G, Mitermayer F, Zorn S, Marsik C, Polterauer S et al. Simvastin prevents vascular hyporeactivity during inflammation. Circulation 2004;110:3349-3354
33 Dekkers PE, Juffermans NP, Hove T, de Jonge E, van Deventer SJ, van der Poll T. Granulocyte colony-stimulating factor receptors on granulocytes are down-regulated after LPS administration to healthy humans. I Infect Dis 2000;181:2067-2070
34 Juffermans NP, Dekkers PE, Verbon A, Speelman P, van Deventer SJ, van der Poll T. Concurrent upregulation of urokinase plasminogen activator receptor and CD11b during tuberculosis and experimental endotoxemia. Infect Immun 2001;69:182-185
35 Hirsh M, Mahamid E, Bashenko Y, Hirsh I, Krausz MM. Overexpression of the high-affinity Fcgamma receptor (CD64) associated with leukocyte dysfunction in sepsis. Shock 2001;16:102-108
36 Wyllie DH, Bowler IC, Peto TE. Bacteraemia prediction in emergency medical admissions: role of C reactive protein. J Clin Pathol 2005;58:352-356

Chapter 10

Measurement of neutrophil membrane cd64 and hla-dr in a patient with abdominal sepsis

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J Infect *In press*

Abstract
A patient with abdominal sepsis had both intra and extracellular bacteria in a blood smear and high levels of neutrophil membrane CD64 and HLA-Dr. Intracellular bacteria are only observed in the terminal phase of a sepsis. Our patient recovered, suggesting that a high expression of neutrophil CD64 is indicative for a good prognosis.

Case report and methods

A 3-year-old boy treated for acute lymphoblastic leukaemia, developed a perforation of the distal oesophagus during chemotherapy. He developed severe oesophageal stenosis and a tracheo-oesophageal fistula. Associated feeding difficulties and recurrent lower respiratory tract infections required surgical correction by oesophagectomy and insertion of a gastric tube. Seven days later he developed severe pancolitis, which was treated with total parenteral feeding and metronidazole. On day 14, while in intensive care, he developed septic shock and respiratory insufficiency. Haematological investigations revealed anaemia (Hb 4.9 mmol/L) with normal platelet (347 $x10^9$/L) and white blood cell (9.9 $x10^9$/L) counts. The leukocyte differential comprised 5% metamyelocytes, 20% non-segmented (band) neutrophils, 58% segmented neutrophils, 13% lymphocytes and 4% monocytes. Intra-neutrophilic and extracellular bacteria as well as neutrophil toxic changes (*figure 1*) were also noted.

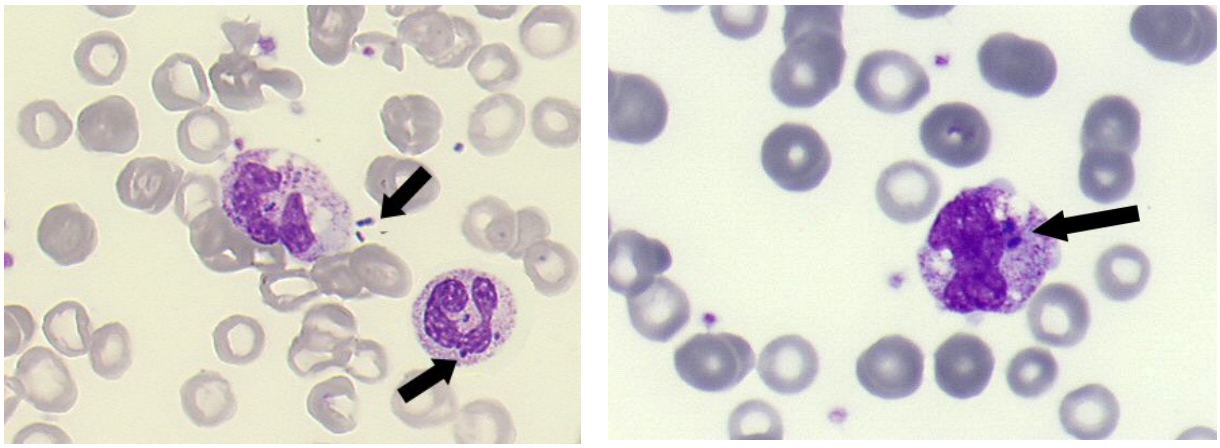


fig 1
(a) Peripheral blood neutrophils showing both intra-neutrophilic and extracellular bacteria(arrows). (b) Neutrophil with internalised bacteria (arrow) and toxic changes (hypergranularity and vacuolation).

Other laboratory findings included a C-reactive protein of 229 mg/L (normal <5 mg/L), fibrinogen 5,910 mg/L (normal 1,500-3,500 mg/L) and D-dimer 15,970 ng/mL (normal <500 ng/mL). A diagnosis of bacterial

septicaemia with secondary disseminated intravascular coagulation (DIC) was made and broad-spectrum antibiotics (meropenem and tobramycin) were started after collection of blood, urine and tracheal secretions for culture. He was intubated because of respiratory failure and the port-a-cath was removed as it was considered the likely source of the bacteraemia. A laparotomy was also performed because of suspected intestinal perforation and large amounts of ascites were removed. The small intestines were vital with no abnormalities, and the colon showed signs of pancolitis without a perforation. The abdomen was left open to ensure efficient drainage. Blood cultures from the port-a-cath and from peripheral veins revealed *Enterobacter aerogenes* and *Enterococcus faecalis*. The following day, renal failure and liver function abnormalities developed together with persistent lactic acidosis, hyperglycaemia and hypertension. High ventilation pressures were required to induce adequate oxygenation. Further haematological investigations showed a slight increase in the granulocyte count (to 9.0 x 10^9/L) and moderate thrombocytopenia (97 x 10^9/L). Four days after laparotomy, his clinical condition improved and enteral feeding by jejunostomy was started. Renal and liver function improved, CRP decreased to normal levels, platelet count and DIC recovered, and the fever disappeared. Extubation followed after 7 days of artificially ventilation. He was treated for 2 weeks with meropenem and teicoplanin. Three weeks later, a Ramirez abdominoplasty was performed and a perforation of the colon found and attributed as the focus of his poly-microbial septicaemia. At present, the boy is doing relatively well.

In addition to standard laboratory investigations, an analysis of neutrophil membrane CD64 and HLA-Dr (Ia) expression was undertaken in a blood sample taken at the initial sepsis work-up at the moment of clinical presentation of bacteraemia. Measurement of neutrophil membrane CD64 (PMN-CD64) has been the subject of recent reports[1, 2] and is considered potentially useful for the diagnostic assessment of sepsis for patients when conventional haematological parameters may be uninformative[3]. The CD64 antigen is a high-affinity receptor (FcγRI) expressed by monocytes but not by normal neutrophils. However, upregulated and detectable expression of PMN-CD64 is mediated by lipopolysaccharides, γ-interferon and granulocyte colony-forming factor (G-CSF)[4], and is also characteristically seen in patients with infection. In contrast, constitutively expressed membrane Ia by immature granulocytes is effectively lost with neutrophil differentiation from the myelocyte.

The immunofluorescent detection and quantitation of neutrophil CD64 and Ia was performed using the Cell-Dyn CD4000 haematology analyser (Abbott Diagnostics, Santa Clara, CA, USA) as previously described[5].

Patient expression of neutrophil CD64 and Ia was compared to a series of 10 healthy individuals (4 males, 6 females; mean age 21, range 18-24 years, without febrile illness or medication in the 2 weeks preceding the study). These studies revealed that granulocytes in our patient had a markedly increased median expression of CD64 of 750 AFU (Arbitrary Fluorescent Units) compared to a normal median of 98 AFU (SD 8.7) (*figure 2*). Expression of membrane Ia (*figure 2*) was also significantly increased (26.4 AFU) compared to normal (median 10.7, SD 0.6) although this level of expression is relatively low compared to monocyte expression, which is typically in excess of 260 AFU[5].

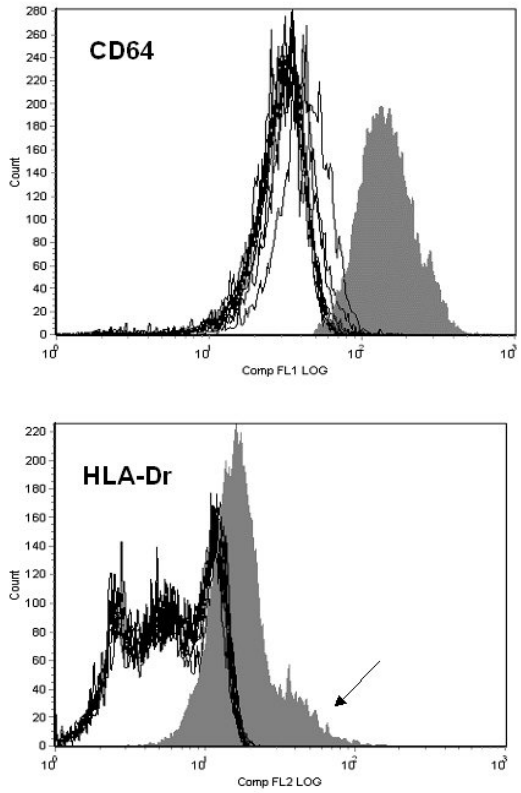


fig 2
Quantitative staining intensities for neutrophil membrane CD64 and HLA-Dr (Ia) expression. Neutrophils were gated according to optical scatter characteristics (0º versus 7º), and post-acquisition fluorescent channel compensation was then applied. The histogram displays of FL1 (CD64) or FL2 (Ia) staining for our patient (shaded) are compared to those obtained from 10 normal control samples. Neutrophil CD64 staining profiles were normally distributed while Ia expression was typically non-normal. Note the markedly increased CD64 and the weakly increased Ia expression. The minor population of granulocytes in our patient (arrow) with higher Ia staining may correspond to the small proportion of immature granulocytes (metamyelocytes) noted in the morphological differential.

Discussion

The expression of neutrophil CD64 was very high in our patient at the time of clinical presentation with bacteraemia. In a previous study of 117 patients, comprising patients with and without known infections, using the same assay procedure[5], only six were found with a neutrophil CD64 level exceeding 700 AFU, with the mean CRP values for these being 260 mg/L (range 176-360 mg/L), indicating that a high CRP value is associated with a high CD64 level. Determination of neutrophil membrane Ia expression has not been systematically looked at previously although one report found that increased Ia expression could

be induced in some patients treated with γ-interferon[6]. We took the opportunity to analyse this in our patient and found a modest but nevertheless significant increase compared to normal. To establish whether this could be a consistent phenomenon, neutrophil Ia expression was retrospectively determined for the six previously investigated samples with high neutrophil CD64. The mean neutrophil Ia level for these was 21.7 AFU (observed 18.4 – 25.4 AFU) and thus similar in magnitude to that seen with our patient. While these higher values could reflect the presence of immature components, as our patient indeed had a leukocyte differential that included 5% metamyelocytes and 20% band cells, this is considered unlikely as only three of these six patient samples did show immature granulocytes[5]. Disordered granulopoiesis, such as cellular asynchrony, might also be proposed as a reason for high neutrophil CD64 expression in patients with leukaemia although again unlikely as the primary malignancy in our patient was lymphoid in nature and at the time of this episode of bacteraemia the leukaemia was in remission.

Excluding samples with post-venesection contamination, intracellular bacteria are only observed in blood smears in the terminal phase of sepsis[7-9]. Earlier reports suggest that neutrophil CD64 expression can be used for diagnostic assessment of sepsis[2, 10], and that a poor prognosis is associated with a lower neutrophil CD64 (and monocyte Ia) expression[11, 12].
With regards to monocyte Ia, significant reductions are characteristically seen in patients with sepsis[11, 13, 14] with reversion to normal levels of expression as sepsis-associated transient immunosuppression is overcome[15]. However, this measurement can be problematic in patients when severe monocytopenia occurs during the acute stages of sepsis. Analysis of neutrophils in such circumstances thus offers a number of advantages. We therefore evaluated a new and simple approach for measuring these cellular antigens with a routine haematology analyzer and confirmed markedly elevated CD64 expression in our patient, and additional evidence for mildly upregulated membrane Ia. While the diagnosis in our patient was not in doubt, these observations nevertheless suggest that such measurements could be useful in investigating patients where a suspicion of sepsis is more equivocal.

Acknowledgment
The authors like to thank Ms Jacqueline Dinnissen for interpretation of blood smears.

References

1 Davis BH, Bigelow NC, Curnutte JT, Ornvold K. Neutrophil CD64 expression. Potential diagnostic indicator of acute inflammation and therapeutic monitor of interferon-γ therapy. Lab Hematol 1995;1:3-12

2 Ng PC, Li G, Chui KM, Chu WCW, Li K, Wong RPO, et al. Neutrophil CD64 isa sensitive diagnostic marker for early-onset neonatal infection. Pediatr Res 2004;5:796-803

3 Shapiro MF, Greenfield S. The complete blood count and leukocyte differential count. An approach to their rational application. Ann Intern Med 1987;106:65-74

4 Schiff DE, Rae J, Martin TR, Davis BH, Curnette JT. Increased phagocyte Fc gammaR1 expression and improved Fc gamma-receptor-mediated phagocytosis after in vivo recombinant human interferon-gamma treatment of normal human subjects. Blood 1997;90:3187-3194

5 Van der Meer W, van Dun L, Klein Gunnewiek J, Scott CS. Simultaneous determination of membrane CD64 and HLA-DR expression by blood neutrophils and monocytes using the monoclonal antibody fluorescence capability of a routine haematology analyser *accepted J Immunol Methodod*

6 Reinisch W, Lichtenberger C, Steger G, Tillinger W, Scheiner O, Gangl A, et al. Donor dependent, interferon- γ induced HLA-DR expression on human neutrophils in vivo. Clin Exp Immunol 2003;133:476-484

7 Lehmann LS, Spivak JL. Rapid and definitive diagnosis of infectious diseases using peripheral blood smears. J Intensive Care Med 1992;1:36-47

8 Van der Meer W, Verwiel JMM, Gidding CEM, de Metz M, de Keijzer MH. Bacteria in blood smears: overwhelming sepsis or trivial contamination. Acta Haematologica 2002;107:220-223

9 Blot F, Nitenberg G, Chachaty E, Raynard B, Germann N, Antoun S, et al. Diagnosis of catheter-related bacteraemia: a prospective comparison of the time to positivity of hub-blood versus peripheral-blood cultures. Lancet 1999;354:1071-1077

10 Hodge G, Hodge S, Han P, Haslam R. Multiple leukocyte activation markers to detect neonatal infection. Clin Exp Immunol 2004;135:125-129

11 Muller Kobold AC, Tulliken JE, Zijlstra JG, Sluiter W, Hermans J, Kallenberg CGM, et al. Leukocyte activation in sepsis; correlations with disease state and mortality. Intensive Care Med 2000;26:883-892

12 Volk HD, Reinke P, Krausch D, Zuckermann H, Asadullah K. Muller JM et al. Monocyte deactivation—rationale for a new therapeutic strategy in sepsis. Intensive Care Med 1996;22:S474-481

13 Le Tulzo Y, Pangault C, Amiot L, Guilloux V, Arvieux C, Camus C, et al. Monocyte human leukocyte antigen-DR transcriptional downregulation by cortisol during septic shock. Am J Respir Crit Care Med 2004;169:1144-1152

14 Sedlackova L, Prucha M, Dostal A. Immunological monitoring of sepsis using flow cytometry–quantitation of monocyte HLA-DR expression and granulocyte CD64 expression. Epidemiol Mikrobiol Immunol 2005;54:54-61

15 Drossou-Agakidou V, Kanakoudi-Tsakalidou F, Sarafidis K, Tzimouli V, Taparakou A., Kremenopoulos G, Germenis A. In vivo effect of rhGM-CSF and rhG-CSF on monocyte HLA-DR expression of septic neonates. Cytokine 2002;18:260-268

Chapter 11

Summary & conclusions

Summary

In this thesis we describe controversial morphologic features in both microscopic and automated differentiation of blood cells. In addition, we have investigated alternative methods to overcome these shortcomings. Furthermore we describe the variance of microscopic counting of band cells and variant lymphocytes. We also describe the automated measurement of platelets in acute leukaemias. Finally, we describe alternatives for sepsis diagnosis.

In Chapter 2 we describe the presence of bacteria in blood smears observed by microscopy. These bacteria were not yet defined using standard microbiological methods. This early detection of bacteria may have major therapeutic consequences. Presence of bacteria in blood smears from asplenic patients, especially *Capnocytophaga canimorsus,* may have a dramatic outcome, which may have major therapeutic consequences. Therefore it is important to investigate their origin of presence. These bacteria may be a result of a fulminant sepsis, catheter related infection, or contamination. Intra- and/or extra cellular bacteria are frequently found in patients with a central venous catheter (CVC), and may point to a catheter related infection. Since blood samples of these patients are obtained by the CVC, blood sampling by finger puncture may exclude a catheter related infection. If in these samples no bacteria are found the CVC should be removed since removal leads to recovery of the infection. Bacteria as result of contamination may appear both intra- and extracellular. If additionally undefined coloured pieces are seen in the blood smear, filled with similar bacteria, these are likely to be due to *in vitro* contamination.

Automated analysers may generate flaggings in case of suspicious abnormal cells. In Chapter 3 we describe the overestimation of atypical lymphocytes or left shift in case of flagging for these cells. Five slides with a various amount of atypical lymphocytes and five slides with different amount of band cells were observed by 30 technicians of three laboratories. No supplemental information was given. After a period of two months the same slides were observed by the same technicians, but now accompanied by supplemental haematological information. Flagging for atypical lymphocytes resulted in an overestimation of atypical lymphocytes, whereas in flagging for left shift no overestimation was achieved. This may be due to the enormous intervariation of band cell counting.

In Chapter 4 we describe this variation of band cell enumeration. This was tested among Dutch hospital laboratories. Blood of a septic patient was used to obtain a blood smear. At random 100 white blood cells were micro photographed. These images were processed in a PowerPoint presentation. The PowerPoint presentation was sent out to 157 laboratories with the request to differentiate the cells. Eighty-six percent of the laboratories responded (756 individuals) and the results were compared. For band cells a SD was found of 11.0% in a range of 4-64%. Segmented neutrophils revealed a SD of 11.0% in a range of 15-72%. One year later a second PowerPoint presentation of the same patient was sent. This time we requested to differentiate cells but not to differentiate between band cells and segmented neutrophils. Seventy-three percent of the laboratories responded (637 individuals) and resulted in a SD of 2.0% for the neutrophils with a range of 59-77%.

Abnormal lymphocytes may appear in different diseases as well as an artefact. In chapter 5 the divergent assessment of atypical lymphocytes is described. An abnormal morphology of lymphocytes may be suggestive for infection or a (premalignant) neoplasm. However, morphology of lymphocytes is difficult to interpret. Of a patient with a mild lymphocytosis (56% lymphocytes), which was normal for the patients age, 100 at random cells were micro photographed and processed in a PowerPoint presentation. This PowerPoint presentation was sent to 157 Dutch hospital laboratories. They were requested to differentiate the blood cells. Moreover, the participants were asked to differentiate lymphocytes in normal, atypical, plasma cells, prolymphocytes or (lympho)blasts. The response was 73% (671 individuals). For only 7 cells (normal lymphocytes) a concordance of >90% was reached, which mean that more than 90% of the observers called these cells a normal lymphocyte. With respect to the other 49 cells there was no concordance at all. Classification resulted in various types of lymphocytes. One cell was shown twice in the PowerPoint presentation. Twohundred-ten out of 671 persons classified this same cell as two different subtypes. Nevertheless, reporting atypical lymphocytes may contribute to a rapid diagnosis of storage diseases, which is described in Chapter 6. Automated flagging for atypical lymphocytes lead to a microscopic observation of the blood smear. In this smear abnormal lymphocytes were observed. These lymphocytes differed from reactive or neoplastic lymphocytes. Vacuole like inclusion bodies were seen and were suggestive for a rare autosomal storage disease, the so-called I-cell disease. The diagnosis was confirmed by electron microscopy and lysosomal enzyme levels in fibroblasts and serum.

In the past, microscopic assessment of platelet morphology was used. However, nowadays it is not used since it is labour intensive and imprecise. Microscopic observation of platelet morphology however, may be useful in cases where spurious thrombocytosis can be expected. In Chapter 7 we describe the incidence of spurious platelet counts in acute leukaemias. In acute leukaemia, platelet-sized particles may be present in the blood. In a retrospective study of 169 patients, pseudoplatelets were found in 43 patients. Seven patients were even re-classified as having a major bleeding risk, which means they have a true platelet count of less than $15x10^9$/L.

Sepsis can lead to septic shock and death. The incidence of sepsis is about 95 cases per 100,000 patients in the United States of America. Because mortality increases with progressive sepsis, early detection of sepsis is very important. Haematological, biochemical and immunological analyses are variously used as sepsis parameters. Expression of CD64 (high affinity FcγRI receptor) on neutrophils (nCD64) has been the subject of recent research. In Chapter 8 a new method is described to measure neutrophil nCD64 and HLA-DR in an easy way on a routine haematology analyser. The Cell Dyn 4000 (Abbott Diagnostics) can be expanded with a special module to measure the monoclonals CD4 and CD8. Using this module nCD64 and HLA-DR can be analysed. Multiple plot files can be generated with parameter options being 7º intermediate angle scatter, 0º Axial Light Loss (size), FL1, FL2 and FL3. Measurement of neutrophil CD64 and monocyte HLA-DR as described in this chapter overcomes a number of limitations of flow cytometry. The use of nCD64 is described in Chapter 9. During experimental human endotoxemia the modulation of nCD64 expression is examined and compared with other sepsis parameters such as haematological indices, CRP, cytokines and interleukins.
In Chapter 10 a patient is described suffering from abdominal sepsis with both intra and extracellular bacteria in the blood smear. This patient had high levels of nCD64 as well as high levels of HLA-DR expression on neutrophils. Normally, intracellular bacteria are only observed in the terminal phase of sepsis. Since the described patient recovered, we hypothesize that a high nCD64 expression might be indicative for a good prognosis.

Conclusions and prospectives

Microscopic differentiation of blood cells is a time consuming and inaccurate laboratory test, which requires expertise and experience. The inaccuracy is due to the small amount of cells, which is counted. Automated differentiation saves a lot of time and the obtained results are statistically more reliable. However, the flagging for abnormalities, shows less specificity and leads to overestimation of abnormalities. The technician usually does not have any clinical indication for a differential. This may result in unnecessary information for the clinician, e.g. presence of atypical lymphocytes in patients with a diagnosed viral infection does not provide essential information. Therefore most of the microscopic observations are superfluous and the differentials must be reduced.
Automated flagging for band cells (left shift) should not be evaluated by microscopic observation. Because of the enormous variation in quantification of band cells, and the huge intra- and intervariation, reporting of absolute neutrophil count is sufficient. Additional laboratory parameters such as C-reactive protein (CRP) and procalcitonin (PCT) provide more information in addition to the absolute neutrophil count. Interleukins and cytokines may provide additional specific information.
Neutrophil CD64 expression is a promising cellular sepsis marker, which can be measured using a haematology analyser (Cell Dyn 4000). Moreover, a high expression of neutrophil CD64 might be suggestive for a good prognosis in bacteraemia.
In this thesis we also show that morphology of atypical lymphocytes without any clinical information leads to confusion while morphology is indistinct. Therefore, morphologic evaluation of lymphocytes should only be performed when accompanying clinical information is present.

The development of new haematology instruments with expandable possibilities, e.g. analysis using monoclonals like neutrophil CD64, will lead to a further decrease in microscopic blood cell morphology. In addition, the development of automated cell recognition systems will make manual microscopy redundant. Both developments will lead to a reduction of the microscopic blood cell differentiation in the future. Therefore, in laboratories, a small group of experts should be formed and well trained by means of educational programmes and national quality assessments.

Although controversies in microscopic examination of blood cells are profound, in some cases it is still required. In 2000 the Dutch Association of Laboratory Haematology Diagnosis (Vereniging Hematologische

Laboratoriumdiagnostiek; VHL) presented recommendations concerning the use of microscopic differentiation of blood cells. In their conclusions, which are mainly based on stat indications, reduction of microscopic differentiation of white blood cells should be established. We recommend to perform a microscopic white blood cell differentiation, whether emergency or not, in the next cases:

- In acute leukaemia in order to exclude pseudothrombocytosis
- If inexplicable diseases are present which can not be explained by other laboratory diagnosis
- If haematology analysers show ambiguous interpretable scatterplots
- In case of possible pseudothrombopenia

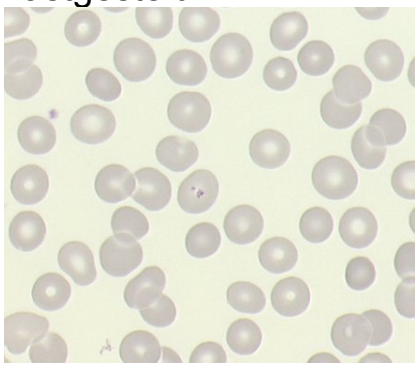
When microscopic examination of white blood cells is necessary (indicated) a screening for abnormalities is sufficient. In that case microscopic white blood cell differentiation is not necessary at all.

Chapter 12

Nederlandse samenvatting en conclusies

Bloedcellen

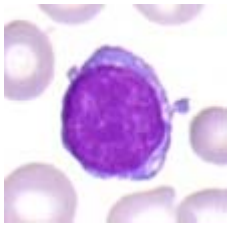
In één liter bloed bevinden zich ongeveer $4x10^{12}$ rode bloedcellen (erytrocyten), die verantwoordelijk zijn voor het zuurstoftransport. Als er te weinig voedingsstoffen (zoals ijzer) worden ingenomen heeft dat gevolgen voor de aanmaak van deze rode bloedcellen. Men spreekt dan van bloedarmoede (anemie). In het laboratorium kan anemie worden vastgesteld.



rode bloedcellen

Daarnaast bevat het bloed witte bloedcellen (leukocyten), die zorgen voor de afweer tegen onder andere bacteriën en virussen. Eén Liter bloed bevat ongeveer $10x10^{9}$ witte bloedcellen. Maar ook op ander plaatsen in het lichaam komen witte bloedcellen voor. De witte bloedcellen zijn divers met verschillende functies. De granulocyten zorgen voor de bacteriële afweer, terwijl de lymfocyten voor de virale afweer (bv een verkoudheid) zorgen. Een eosinofiele granulocyt is vooral zichtbaar bij hooikoorts en andere allergieën.

De verschillende witte bloedcellen



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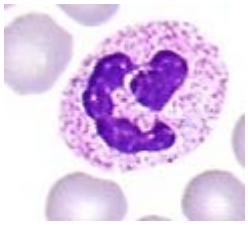

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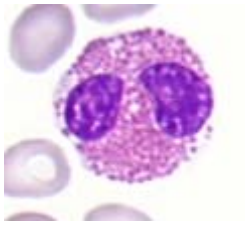
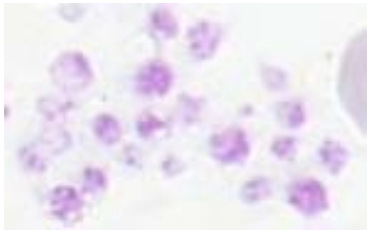


eosinofiele granulocyt

Tenslotte bevat het bloed ook bloedplaatjes (trombocyten). Deze kleine cellen zijn noodzakelijk voor de bloedstolling. Bij het ontstaan van een wond plakken bloedplaatjes aan elkaar (aggregeren) aan de wondrand. Deze aggregaatvorming stimuleert de bloedstolling. Er circuleren ongeveer $150x10^{9}$ bloedplaatjes in één liter bloed.



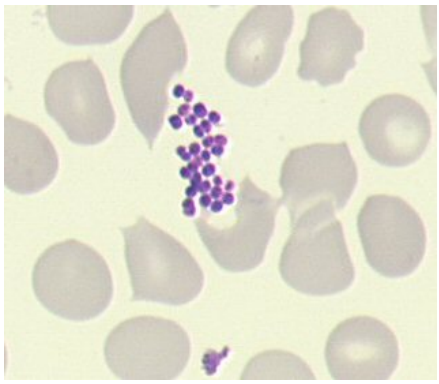
bloedplaatjes

De bloedcellen (gemiddelde grootte is 0,007 mm) kunnen onder een microscoop bekeken worden. Hiervoor wordt een druppel bloed uitgestreken op een glaasje waardoor een heel dunne bloedfilm ontstaat. Bij ziekte kan de hoeveelheid bloedcellen of de verdeling van de bloedcellen verstoord zijn. Daarom worden deze tellingen vaak uitgevoerd in laboratoria.

Automatische celtellers

Naast de microscoop kunnen ook automatische celtellers gebruikt worden. Cellen worden al sinds decennia geteld op automatische bloedceltellers en tegenwoordig zijn de celtellers in staat de verschillende soorten cellen te herkennen (differentiatie). De kwaliteit van deze celtellers is dusdanig dat ze nauwkeurigere resultaten geven dan analyses met behulp van de microscoop. Bovendien is de apparatuur zo gevoelig dat deze vaak een signaal geeft voor een abnormaliteit zonder dat er sprake is van een afwijking. Het komt echter ook voor dat bepaalde afwijkingen niet gezien worden.

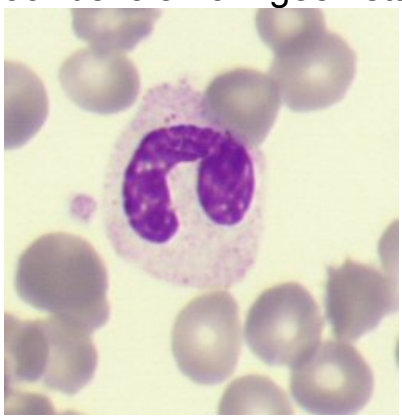
In hoofdstuk 2 worden 4 cases besproken waarbij bacteriën in het bloed aanwezig zijn. Dit kan duiden op een ernstige bloedvergiftiging (sepsis), of het gevolg zijn van een katheter gerelateerde ontsteking. Echter, deze bacteriën kunnen ook het gevolg zijn van vervuiling na het prikken. Omdat aanwezigheid van bacteriën een ernstige zaak is, is het belangrijk om te weten of er sprake is van sepsis, ontsteking of verontreiniging. Automatische celtellers herkennen geen bacteriën in het bloed. Echter, meestal gaat een ernstige sepsis gepaard met andere afwijkingen waarvoor de automatische celteller wel een alarm geeft. Indien bacteriën gevonden worden in een bloeduitstrijkje is het dus altijd belangrijk eerst te onderzoeken of er sprake is van verontreiniging of sepsis.



bacteriëmie

De automatische bloedceltellers kunnen aangeven of er een verdenking is van aanwezigheid van afwijkende cellen. Het kan ook leiden tot een onjuiste inschatting van een afwijking omdat de beoordelaar met de informatie van de automatische celteller eerder geneigd is de gemelde afwijking te zien. In hoofdstuk 3 wordt beschreven dat alarmering voor atypische lymfocyten (abnormale lymfocyten) door de automaat leidt tot overschatting van atypische lymfocyten in een bloeduitstrijkje. Alarmering voor een zogenaamde linksverschuiving (aanwezigheid van jonge granulocyten) leidt daarentegen niet tot overschatting. Bij dit onderzoek zijn 5 gekleurde bloeduitstrijkjes, met wisselende hoeveelheid atypische lymfocyten, door 30 analisten uit 3 ziekenhuizen beoordeeld. Ook zijn 5 gekleurde bloeduitstrijkjes met wisselende hoeveelheid staafkernige granulocyten meegestuurd. Er werden geen hematologieuitslagen vermeld. Na 2 maanden werden dezelfde uitstrijkjes nog eens ter beoordeling aangeboden. Nu gingen de uitstrijkjes vergezeld van informatie zoals alarmering voor atypische lymfocyten en/of aanwezigheid van jonge granulocyten. De resultaten wezen uit dat alarmering voor atypische lymfocyten leidt tot een overschatting van het aantal atypische lymfocyten. Vreemd genoeg is dat niet het geval voor staafkernige granulocyten. Dat wordt waarschijnlijk veroorzaakt door de grote variantie in het tellen van staafkernige granulocyten.
In hoofdstuk 4 is deze bevinding verder uitgewerkt. Van het bloeduitstrijkje van een patiënt met een sepsis (bloedvergiftiging waarbij jonge granulocyten in het bloed verschijnen) zijn 100 microfoto's gemaakt en in een PowerPointpresentatie gezet. Deze presentatie is op cd-rom gezet en verstuurd naar 157 ziekenhuislaboratoria met het verzoek om de cellen te differentiëren. 106 Laboratoria stuurden hun resultaten in met 756 individuele beoordelingen. Zowel het laboratoriumgemiddelde als het individuele gemiddelde werd verwerkt. Het gemiddeld gevonden aantal staafkernige granulocyten was 31% met een spreiding van 4-64%! Voor de segmentkernige granulocyten werd

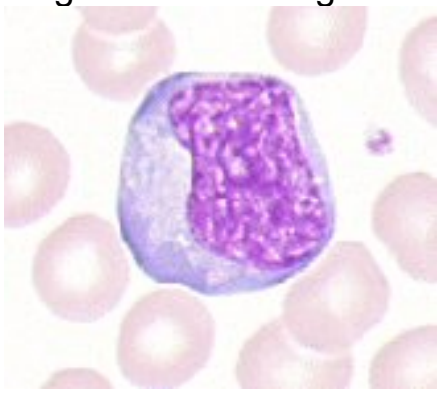
een gemiddelde gevonden van 44% met een spreiding van 15-72%. Van dezelfde patiënt werd na 1 jaar een nieuwe set beelden gemaakt en naar dezelfde laboratoria gestuurd. Echter, deze keer werd verzocht om geen onderscheid te maken tussen staafkernige en segmentkernige granulocyten. Van de 157 laboratoria werden door 114 laboratoria resultaten ingestuurd met een totaal van 637 individuele resultaten. Het gemiddelde aantal granulocyten bedroeg 70% met een spreiding van 59-77%. Dat men moeite heeft om van de staaf afscheid te nemen bleek uit het feit dat op veel formulieren toch werd aangegeven hoeveel staafkernige granulocyten aanwezig waren. Gezien de geringe klinische betekenis van de staafkernige granulocyt en de enorme spreiding wordt aanbevolen om geen staafkernige granulocyten meer te rapporteren.



staafkernige granulocyt

De morfologie van lymfocyten kan bijdragen tot een snelle diagnose van een kwaadaardige ziekte of een infectie met een virus. Soms kan ook een zeldzame afwijkingen gediagnosticeerd worden. Echter, de beoordeling van afwijkende lymfocyten is nogal divers. In hoofdstuk 5 is de diversiteit in het beoordelen van lymfocyten onderzocht. Van het bloed van een patiënt met veel lymfocyten werd een uitstrijkje gemaakt en gekleurd. 100 beelden werden gefotografeerd en in een PowerPointpresentatie gezet. Dit werd aan 157 laboratoria gestuurd met de vraag of een differentiatie uitgevoerd kon worden, waarbij bovendien aandacht voor lymfocyten werd gevraagd. Lymfocyten dienden onderscheiden te worden in normaal, atypisch, jong, plasmacel of blast. Er was een respons van 73% met een totaal van 671 individuele resultaten. Het gemiddelde aantal lymfocyten bedroeg 56%. Van deze 56% werden slechts 7 cellen door meer dan 90% van de personen als normaal beoordeeld. Voor 49 cellen werden verschillende beoordelingen gevonden: 30 cellen kregen twee verschillende beoordelingen, 11 cellen drie, voor 7 cellen 4 en voor 1 cel zelfs 5. Eén cel kwam 2 keer voor in de presentatie. Maar liefst 210 personen (van de 671) beoordeelden de cel verschillend! Dit maakt het probleem van de beoordeling van lymfocyten duidelijk. Zonder extra informatie (anamnese, vraagstelling)

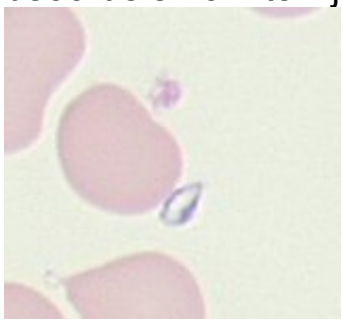
kan het beoordelen van lymfocyten tot verwarring en dus vertraging van de diagnose leiden. Dat het vaststellen van afwijkende lymfocyten wel degelijk belangrijk is, blijkt uit de casus die beschreven is in hoofdstuk 6. Een zeldzame overerfbare stapelingsziekte werd eerder ontdekt dankzij de beschrijving van afwijkende lymfocyten. Dit leidde tot vervolgonderzoek waaronder electronenmicroscopie en meting van lysosomale enzymen. Aan de hand van deze resultaten werd de diagnose definitief gesteld.



Atypische lymfocyt

Bloedplaatjes (trombocyten) worden tegenwoordig altijd automatisch geteld. Microscopische telling van bloedplaatjes met behulp van een telkamer gebeurt slechts zelden. In hoofdstuk 7 wordt de bloedplaatjestelling bij acute leukemie onderzocht. Acute leukemie is een kankersoort waarbij de bloedcellen woekeren. De primaire bron van deze kankervorm is in het beenmerg. De vorming van bloedcellen speelt zich af in beenmerg en het lymfatisch stelsel. Als in het beenmerg cellen ongecontroleerd gaan delen worden, in plaats van rijpe cellen, jonge bloedcellen in het bloed afgegeven. Deze jonge cellen hebben in het bloed geen functie en verdringen de normale cellen. Daardoor ontstaat een tekort aan rijpe bloedcellen. Hierdoor komen zuurstoftransport, afweer en bloedstolling in gevaar. Het tekort aan bloedplaatjes leidt tot langdurige bloedingen. Het aantal bloedplaatjes kan zo ver dalen dat een transfusie met bloedplaatjes gegeven moet worden. Kankercellen in het bloed kunnen cytoplasmafragmenten afsplitsen. Deze kunnen net zo groot zijn als bloedplaatjes. Omdat een hematologieautomaat het verschil niet ziet tussen bloedplaatjes en deze celfragmenten (pseudoplaatjes) zal het aantal automatisch getelde bloedplaatjes hoger zijn dan het in werkelijkheid is. Bij 169 patiënten met een acute leukemie is microscopisch onderscheid gemaakt tussen echte bloedplaatjes en pseudoplaatjes. Bij 43 patiënten zijn pseudoplaatjes gevonden en bij 7 patiënten leidde dat zelfs tot een herclassificatie van het bloedingsrisico. Hierbij is aangetoond dat het belangrijk is om de morfologie van

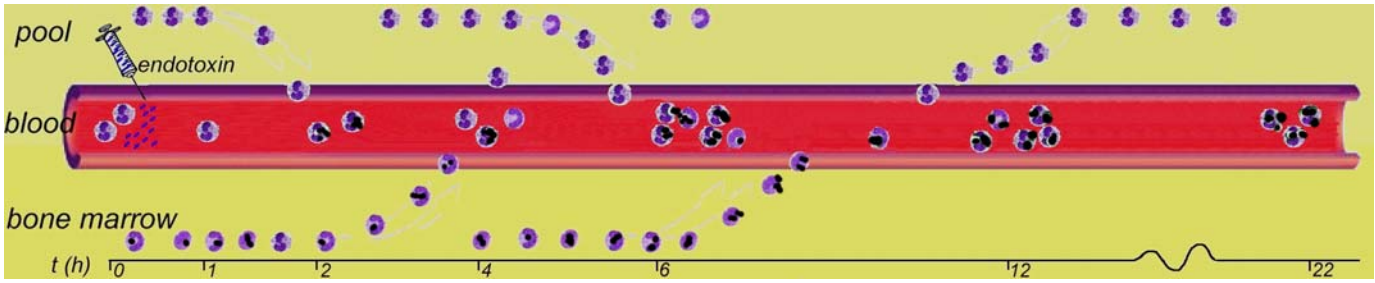
bloedplaatjes bij patiënten met een acute leukemie microscopisch te beoordelen om te kijken of het aantal getelde bloedplaatjes wel klopt.



pseudoplaatjes en trombocyten

Sepsis kan ontstaan als gevolg van infectie en kan leiden tot een septische shock. Dit is een levensbedreigende complicatie. Daarom is het belangrijk de symptomen van sepsis in een vroeg stadium te onderkennen. Soms kan dat aan de hand van een bloeduitstrijkje waar bacteriën gezien worden (hoofdstuk 2). Het is dan eigenlijk al te laat. Er zijn tal van chemische stoffen en cellen bij een sepsis betrokken. Aan- of afwezigheid daarvan kan tot een juiste diagnose leiden. Er is echter (nog) geen ideale sepsismerkstof. De meeste merkstoffen zijn niet specifiek en kunnen ook bij andere aandoeningen voorkomen. Daarbij komt ook dat infectie en/of sepsis een zeer ingewikkeld proces is waar een hele reeks stappen bij betrokken is. Het begint met het herkennen van een ziekteverwekkend element (pathogeen) en via een lange keten van gebeurtenissen zal de gastheer hierop reageren. Herkenning van deze zogenaamde microbe-geassocieerde molecuulpatronen gebeurt door receptoren op cellen en hebben tot gevolg dat allerlei cytokines worden geproduceerd. Cytokines zijn eiwitten die de communicatie tussen afweercellen en andere cellen verzorgen tijdens ontstekingsreacties. Als gevolg van sepsis migreren geactiveerde witte bloedcellen vanuit het weefsel naar de ontstekingshaard. Het beenmerg maakt nieuwe cellen aan waardoor jonge onrijpe cellen in het bloed verschijnen. Dit zijn onder andere de staafkernige granulocyten. In hoofdstuk 5 is al aangetoond dat telling van deze cellen op problemen stuit. Andere merkstoffen zoals ontstekingseiwitten (bijvoorbeeld C-reactief eiwit; CRP) zijn niet specifiek voor sepsis en kunnen bij tal van andere ziekten een rol spelen. Andere specifieke eiwitten, zoals cytokines, zijn vaak moeilijk te meten en kosten veel geld. Geactiveerde granulocyten hebben een eiwit aan de buitenkant van de cel (CD64) dat dient om lichaamsvreemde stoffen te herkennen en ervoor te zorgen dat deze opgeruimd worden. Dit eiwit komt alleen voor op granulocyten die zijn geactiveerd door cytokines. In hoofdstuk 8 wordt een methode beschreven die het mogelijk maakt dat met een hematologie-automaat

(Cell Dyn 4000 met monoclonalenmodule) op eenvoudige en snelle wijze CD64 gemeten kan worden.
Wat de rol van CD64 is in vergelijking met de andere sepsismerkstoffen wordt in hoofdstuk 9 beschreven. Hierbij is uitgegaan van een experimenteel sepsismodel waarbij proefpersonen endotoxine (lipopolysaccharide) ingespoten kregen en op verschillende tijdstippen bloed werd afgenomen. Hiervan werden tellingen verricht van de verschillende bloedcellen en werden cytokines en andere ontstekingseiwitten gemeten en de hoeveelheid CD64 bepaald. Hoewel cytokines als eerste stijgen blijkt ook CD64 snel te stijgen. De stijging van CD64 gaat geleidelijk en in twee fasen. De eerste fase loopt parallel aan de cytokines en de tweede fase loopt parallel aan het CRP. Verder onderzoek moet uitwijzen of deze methode geschikt is als routinebepaling. De rol van CD64 is ook duidelijk gebleken bij ernstige sepsis met bacteriën in het bloed (bacteriëmie). In hoofdstuk 10 is een patiënt met een ernstige bacteriëmie beschreven. Bloed werd afgenomen en in het uitstrijkje waren microscopisch veel bacteriën te zien. Tevens is de CD64-activiteit gemeten en deze bleek zeer hoog te zijn. Ondanks het feit dat bacteriën in bloed een slecht vooruitzicht geeft (zie ook hoofdstuk 2), herstelde de patiënt goed. Dit kan erop wijzen dat een bacteriëmie in combinatie met een hoge CD64-activiteit een gunstig vooruitzicht heeft op genezing.



reactie van granulocyten op endotoxinetoediening

segmentkernige granulocyt

staafkernige granulocyt

segmentkernige granulocyt met CD64-activiteit

staafkernige granulocyt met CD64-activiteit

onrijpe granulocyt met CD64-activiteit

Dankwoord

‘Soms zegt een blik in een bloeduitstrijkje meer dan uitslagen’, met een dergelijk betoog keek Cor van Oostrom regelmatig door de microscoop op het toenmalige Laboratorium Kindergeneeskunde, Afdeling Speciale Hematologie. Met die wijsheid heb ik destijds heel wat uitstrijkjes van bloed en beenmerg bekeken. De eerste stappen geleerd van Cocky Langenhuijsen die met kritische blik uitstrijkjes bekeek en vaak dingen zag waarvoor je een extra zintuig nodig had. Met dr de Vaan, kinderoncoloog, heb ik heel wat gezien. Hij was een vraagbaak van wie ik veel heb geleerd. Eén ding wisten alle wijzen niet: wat zijn toch die kleine op trombocyten lijkende dingen die je soms in bloed en beenmerg ziet. Een lange zoektocht begon en we kwamen uit bij zogenaamde lichaampjes van Hitmair, later bleken ze bekend onder de naam pseudoplaatjes. Als een rode draad koppelde dit fenomeen zich aan mijn morfologie-interesse en uiteindelijk resulteerde dit na jaren tot een artikel (hoofdstuk 7 in dit proefschrift) en kreeg het toch nog een rol in mijn onderzoek. Dr de Vaan zei onlangs nog: “blijf erin geloven, ook al heeft het bij anderen geen prioriteit.” En nog steeds geloof ik dat er te weinig aandacht is voor deze cytoplasmatische afsplitsingen. Morfologie is reuzenspannend: dr de Vaan, Cocky en Cor bedankt voor de basis.

Verder gaat mijn dank uit naar iedereen die aan de totstandkoming van dit proefschrift een bijdrage heeft geleverd. Het is gevaarlijk om namen te noemen, maar toch zal ik een poging wagen.

Als eerste wil ik noemen de collega’s van de Afdeling Klinische Chemie die zich vaak hebben afgevraagd: waar is hij toch mee bezig? Hopelijk is dat nu iets duidelijker. Jacqueline Dinnissen heeft een sleutelpositie ingenomen: loyaliteit, inzet en betrokkenheid bij de, soms tot niets leidende, hersenspinsels waren daarbij essentieel.

Uiteraard bedank ik Hans Willems die mij de ruimte heeft gegeven te promoveren, wat niet zonder risico was. Er moest nog een ‘papiertje’ gehaald worden en wat gepubliceerd worden. Dat allemaal in een voor de afdeling hectische periode.

Dank voor mijn co-promotoren Ries de Keijzer en Jacqueline Klein Gunnewiek. Jullie hulp, enthousiasme, geduld en het vermogen om mij weer met beide benen op de grond te plaatsen als ik weer eens een wild idee had. Om maar te zwijgen van al die taalcorrecties.

Dorine Swinkels ben ik dank verschuldigd. Zij was als klinisch chemica in opleiding betrokken bij mijn eerste onderzoek waarbij zij me leerde om kritisch naar de resultaten te kijken en de onderzoeksopzet. Je was

enthousiast over ‘mijn’ pseudoplaatjes. We hebben tenminste geprobeerd er iets uit te halen.

Ludi van Dun, van de firma Abbott, kwam ik ooit tegen op een symposium van de NVML (Utrecht 1997). Hij was geïnteresseerd in Diffex[1] en vroeg of ik wat plaatjes wilde maken. Daaruit is een hecht contact ontstaan en daar waar mogelijk hebben we elkaar geholpen.

A special word of thank for Steve Scott. You really deserve a statue! You gave me the opportunity to evaluate the CD3700 for the differentials. From that time we had a great cooperation. Four chapters in this thesis are a product of this cooperation! Without Steve this thesis could not exist at all. Breading hours and days on English texts to present data properly, Steve rewrote it in no time in real English. Steve, thank you very much for all your confidence in me!

Jan van den Boogaart van de firma Bayer, wil ik graag bedanken. De vele trucjes die hij mij heeft geleerd om met de ADVIA 120 om te gaan heb ik vaak vergeten, maar telkens weer opnieuw werd het weer uitgelegd door Jan.

Met de Sysmex NE-8000 begon mijn interesse in de automatische morfologie. Jan Willem Schipper en Paul Versteege beiden werkzaam bij Goffin Meyvis, en Jo Linssen van Sysmex Europe, wil ik graag bedanken voor het ontsluieren van de geheimen van de Sysmex NE-8000 en XE-2100. Verdieping in de technologie van de hemocytometrie leidt tot een kritische kijk en onderzoek.

Mijn paranimfen Irma en Erny, leuk dat jullie bereid waren om dingen te doen waarin ik zou falen. Bedankt! Vooral Erny, die het definitieve proefschrift nog eens minutieus heeft gecontroleerd.

Lieve Ingrid, Tijs, Pim, Sjors en Jaap, jullie zijn mijn motivatie om de klus af te maken, bedankt voor jullie steun en vertrouwen.

[1] Diffex: het interactieve morfologieprogramma dat door Felix Cillessen en mij is ontwikkeld

List of publications

Van der Meer W, Pickkers P, Scott CS, van der Hoeven JG, Klein Gunnewiek J. Haematological indices, sepsis markers (crp, tnf-α, ifn-γ, il-6 and il-10) and neutrophil cd64 expression: comparative trends during experimental human endotoxemia. Submitted

Van der Meer W, Koster-Kamphuis L, de Kok JB. Valkuil bij een verworven Pelger Huët. Ned tijdschr Klin Chem. *In press*

Van der Meer W, van Gelder W, de Keijzer MH, Willems JL. The divergent morphological classification of variant lymphocytes in blood smears. J Clin Path. *In press*

Van der Meer W, Scott CS, Verlaat C, Klein Gunnewiek J, Warris A. Measurement of neutrophil membrane cd64 and hla-dr in a patient with abdominal sepsis. J Infect. *In press*

Van der Meer W, van Dun L, Klein Gunnewiek J, Roemer B, Scott CS. Simultaneous determination of membrane CD64 and HLA-DR expression by blood neutrophils and monocytes using the monoclonal antibody fluorescence capability of a routine haematology analyser. J Immunol Methods;311:207-219

Van der Meer W, van Gelder W, de Keijzer MH, Willems JL. Does the band cell survive the 21th century? Eur J Haematol 2006;76:251-254

Ten Brinke MJ, Weerwind PW, Teerenstra S, Ferron JCM, van der Meer W, Brouwer MHJ. Leukocyte removal efficiency of cell-washed and unwashed whole blood: An *in vitro* study. Perfusion 2005;20:335-341

Van der Meer W, Willekens FLA, van Gelder W, Willems JL. De virtuele differentiatie van bloedcellen als hulpmiddel om de controverse van de beoordeling van de staafkernige granulocyt te toetsen. Ned Tijdschr Klin Chem 2004;6:313-316

Van der Meer W, de Keijzer MH. Het automatisch tellen van leukocyten in synoviaalvocht. Tijdschrift BVLT/ABTL 2004;31:187-191

De Keijzer MH, Dujardin BCG, van der Meer W. Een patiënte met een pseudo-verhoogde en EDTA-afhankelijke bezinkingssnelheid. Ned Tijdschr Klin Chem 2004;5:289-290

Van der Meer W, Scott CS, de Keijzer MH. Automated flagging influencing the inconsistency and bias of band cell and atypical lymphocyte morphological differentials. Clin Chem Lab Med. 2004;4:371-377

Van der Meer, W. Band cell and variant lymphocyte estimates: Fact or Fiction? Cell Dyn News Dec.2003:17-18

Van der Meer W, Mackenzie MA, Dinnissen JWB, de Keijzer MH. Pseudoplatelets: a retrospective study of their incidence and interference with platelet counting. J Clin Pathol 2003;10:772-774

Komarova O, Van der Meer W, Levtchenko E, Monnens L. Effective chemical preservation of morphology of urinary erythrocytes. Pediatr Nephrol 2003;7:665-666

Van der Meer W, de Keijzer MH. Het automatisch tellen van leukocyten in synoviaalvocht. Ned Tijdschr Klin Chem 2003;28:157-158

Van der Meer W, Dinnissen JMB, de Keijzer MH. Evaluation of the Sysmex XT-2000i, a new automated haematology analyser. Sysmex Journal International 2002;2:71-75

De Keijzer MH, van der Meer W. Automated counting of nucleated red blood cells in blood samples of new-borns. J Clin Lab Haematol 2002;6:343-34

Van der Meer W, Allebes W, Simon A, van Berkel Y, de Keijzer MH. Pseudothrombocytopenia: report of a new method to count thrombocytes in a patient with EDTA and temperature independent antibodies of the IgM type. Eur J Haematol 2002;69:243-247

Van der Meer W, Dinnissen JWB, de Keijzer MH, Scott CS. Comparative evaluation of Abbott Cell-Dyn CD3700, Bayer ADVIA 120 and Sysmex XE-2100automated leucocyte differentials and flagging. Lab Haematol 2002;8:102

Van der Meer W, Dinnissen JWB, de Keijzer MH, Scott CS. Comparative evaluation of Abbott Cell-Dyn CD3700, Bayer ADVIA 120 and Sysmex XE-2100 automated leucocyte differentials and flagging. Abbott brochure 2001;3

Van der Meer W, Verwiel JMM, Gidding CEM, de Metz M, de Keijzer MH. Bacteria in blood smears: overwhelming sepsis or trivial contamination. Acta Haematologica 2002;107:220-223

Van der Meer W, de Keijzer MH, van Berkel Y, Allebes W. Pseudotrombopenie: altijd weer anders. NVB bulletin 2001;4:35

Van der Meer W, Jakobs BS, Bocca G, Smeitink JAM, Schuurmans Steckhoven JH, de Keijzer MH. Peripheral blood lymphocyte appearance in a case of I cell disease. J Clin Pathol 2001;54:724

Bocca G, Noordam C, Wevers RA, de Jong JGN, van der Meer W, de Keijzer MH, Korver CRW, Smeitink JAM. I-cell disease presenting with severe hypophosphatemia and cardiomyopathy. Neuropediatrics 2000;31:49

Van der Meer W en de Keijzer MH. Drumsticks: meer dan een aanhangsel? Analyse 1999;8:243-244

Van der Meer W, de Keijzer MH. Paracetamol-intoxicatie en vacuolisatie in bloedcellen. Ned Tijdschr Klin Chem 1999;24:354-355

De Keijzer MH, Swinkels DW, van der Meer W. Thrombocytopenia and giant platelets without major hemorrhagic complications in a pregnant patient. Ann Clin Biochem 1998;35 :678

Van der Meer W, Swinkels DW, Willems JL. Automatische detectie van blasten door de Sysmex NE-8000: een vergelijking met de H*1 Technicon. Ned Tijdschr Klin Chem 1998;23

van der Meer W, Swinkels DW, Willems JL. The characterisation of leukaemias with the Sysmex NE-8000. Acta Haematologica 1997;98; 195

Willems JL, van der Meer W. ‘Het differentiëren van witte bloedcellen’; een toetsingsstudie uit het laboratorium. CBO-nieuwsbrief; 1994;14:5-6:

Van der Meer W, Swinkels DW, Willems JL. Changes in the Sysmex NE-8000 most frequent volumes parameters during the course of acute lymphatic leukemia studied in four patients. Sysmex Journal International, Vol.4 No.2 winter 1994

Monnens L, van der Meer W, Langenhuijsen C, van Munster P, van Oostrom C. Platelet aggregating factor in the epidemic form of hemolytic-uremic syndrome in childhood. Clin Nephrol 1985;24:135-137

cd-roms

F.H.J.M. Cillessen en W. van der Meer

Diffex, De digitale dif-expert 1995	ISBN 90 373 0309 9
Atlas of Blood Cell Differentiation 1997	ISBN 0 444 82544 4
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Atlas of Blood Cell Differentiation III 2006	in press

Curriculum vitae

Wim van der Meer werd geboren op 7 juni 1955 in Tilburg. Na het behalen van het MAVO-4 diploma aan de Beatrix-MAVO te Tilburg, begon hij aan de studie voor medisch analist aan de Brabants Medisch Analisten School te Breda. In 1976 haalde hij daar het diploma op niveau HBO-A richting klinische chemie. Er volgde een half jaar studie aan de Botanische Analisten School (STOVA) te Wageningen. Deze studie werd verruild voor een werkplek per februari 1977 op het Laboratorium Kindergeneeskunde en Chirurgie op het toenmalige Sint Radboudziekenhuis. In 1980 specialiseerde hij zich op de morfologie van bloedcellen (inclusief beenmerg en liquor) voor de Afdeling Kinderoncologie en Kindergeneeskunde. Naast speciale hematologische technieken werd ook naar de plaatjes aggregatiefactor (PAF) gekeken bij patiënten met een hemolytisch uremisch syndroom (HUS). Verder werd ook de bepaling van porfyrines op de HPLC opgezet. In 1988 verruilde hij het Laboratorium voor Speciale Hematologie Kindergeneeskunde voor het, als gevolg van reorganisatie, ontstane Centraal Klinsch Chemisch Laboratorium. Onder zijn leiding werd inhoud gegeven aan de microscopische differentiaties en de automatische differentiatie werd ingevoerd. Vanaf 1990 wordt zijn functie uitgebreid met de opleiding van klinisch chemisch analisten (MLO- en HLO-stagiairs) en het inwerken van nieuwe medewerkers. Vanaf 2002 is hij docent op het UMCN, onder andere voor de studie Biomedische Wetenschappen. In 2003 haalde hij zijn bacheloraat aan de Hogeschool Arnhem Nijmegen (HAN). Tevens voert hij naast zijn werkzaamheden promotieonderzoek uit dat zijn bekroning vindt in de onderliggende dissertatie. Met het behalen van dit doel eindigt helaas zijn onderzoekslijn hematologie op de Afdeling Klinische Chemie.